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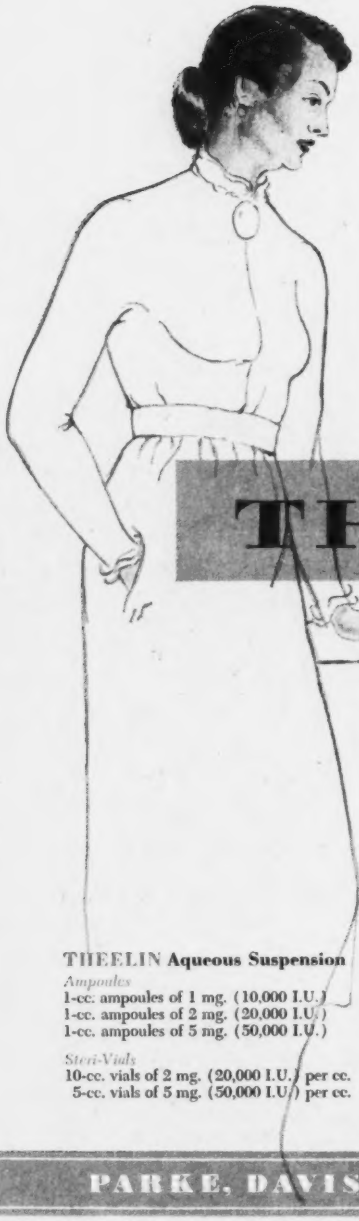

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ENDOCRINOLOGY

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THE EFFECT OF ALLOXAN DIABETES ON THE RESPONSE OF THE ADRENAL GLAND TO COLD STRESS[1]

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THE existence of a functional relationship between the anterior pituitary, the pancreas and the adrenal cortex with respect to the control of carbohydrate metabolism is well established. The role of adrenal cortical hormones in the regulation of carbohydrate metabolism was pointed out by Britton and Silvette (1932). Long and Lukens (1936) demonstrated the improvement in diabetes which results from adrenalectomy and Ingle (1941) established the diabetogenic potency of pure cortical hormones. Although not all details of the mechanisms are known, participation of the adrenal cortex in the control of carbohydrate metabolism is unquestionable.

Recently considerable attention has been given to the response of the adrenal cortex to various types of stress (Selye, 1937; Sayers and Sayers, 1948). Many types of environmental stress, drug or toxin administration or trauma will cause an increase in the activity of the adrenal cortex, associated with which hyperglycemia may be observed. These phenomena have been related to stimulation of the gland by pituitary adrenocorticotrophic hormone by Sayers, Sayers, Fry, White and Long (1943). Thus, the individual roles of the pancreas, pituitary and adrenal glands in the control of glucose metabolism, the response of circulating glucose levels to stress and, therefore, the relationship through glucose metabolism of these three glands to stress reactions seems evident.

Little attention has been paid, however, to the possibility that diabetes may act as a stress which could modify the function of the adrenal cortex.

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[1] Supported in part by Grant No. A-13 from the Division of Research Grants and Fellowships, United States Public Health Service.

Venning and Browne (1947) reported urinary corticoids and 17-ketosteroids to be unchanged in diabetes. An increase in the urinary corticosteroids in diabetic acidosis has been reported by McArthur, Sprague and Mason (1950). Furthermore, withdrawal of insulin from one of their patients resulted in a prompt reduction of circulating eosinophils before a detectable increase in corticosteroid excretion occurred. Hinkle, Conger and Wolfe (1950) subjected diabetic and normal subjects to emotional stress and measured the changes in blood ketone bodies. They observed an increase in circulating ketones which was greater in the diabetic than in the normal subjects, a phenomenon which they interpret to involve the pituitary-adrenal system.

Studies on the effect of experimental diabetes on reproduction (Sinden and Longwell, 1949) have led to a search for mechanisms which are related to the inability of diabetic humans (White, 1946) and animals to reproduce normally, and the possible involvement of the adrenal cortex suggests itself. Venning (1946) reported a considerable increase of urinary corticosteroids during pregnancy, an indication of the modification of the level of activity of that gland during gestation. It is possible that diabetes may constitute a stress which so influences the adrenal gland that it is unable to respond adequately during pregnancy. Consequently, the effect of alloxan diabetes on the ability of the adrenal to respond to cold stress has been studied in non-pregnant, diabetic rats. Similar studies with pregnant animals are in progress.

METHODS

Responsiveness of the adrenal gland to stimulation was determined by measurement of ascorbic acid and cholesterol in the gland. Both were determined because of the possibility that abnormalities of lipid metabolism occasioned by diabetes might result in modification of cholesterol responses which would not be reflected by similar changes in ascorbic acid levels. Furthermore, the possibility that the changes of these two substances as the result of stress may not necessarily be interdependent reactions is indicated by the findings of Long (1947), who reported that ACTH depleted adrenal cholesterol in scorbutic guinea pigs in which the adrenal ascorbic acid concentration was practically zero. Doughaday, Jaffe and Williams (1948) found corticosteroid excretion to be normal in human scurvy.

Albino rats from our own colony, 90 to 120 days of age, were made diabetic by the intravenous administration of 55 mg. of alloxan per kg. of body weight. The successful development of diabetes was determined by the presence of a pronounced glycosuria. The subsequent experiments were done after the animals had been diabetic for 7 to 9 days. Females were used for ascorbic acid determination and males for cholesterol. Ascorbic acid was determined following exposure of the animal to a temperature of 5 to 7° C. for one hour. The rats were exposed at 3 to 5° C. for four hours prior to measurement of adrenal cholesterol depletion.

In a portion of the ascorbic acid experiments the animals were killed by placing a large clamp on the neck which simultaneously broke the neck and occluded the blood flow

from the head region. This method was compared to that of Sayers, Sayers, Liang and Long (1946) in which the rats were anesthetized by the intraperitoneal administration of nembutal before removal of the adrenals. The adrenal glands of the rats killed by the clamping procedure (13 rats) or the anesthetic procedure (9 rats) had mean ascorbic acid values of 356 and 345 mg. per 100 gm. of gland, respectively. All of the rats used for adrenal cholesterol determinations were anesthetized with nembutal prior to removal of the gland. The determinations were done on individual glands. Ascorbic acid was determined by the method of Roe and Keuther (1943) as adapted to adrenal gland analyses by Sayers, Sayers and Woodbury (1948). Total adrenal cholesterol determinations were done by the method of Sperry and Brand (1943) after extraction of the gland by the method of Schoenheimer and Sperry (1934). The animals were fed Purina Laboratory Chow during the period preceding the experiment. A comparison of the response of adrenal cholesterol to cold in fasted and non-fasted normal rats was made. None of the diabetic animals was fasted before the experiments were done.

RESULTS

Groups 1 and 2, Table 1, show the adrenal ascorbic acid levels of normal and diabetic rats kept at room temperature. The mean values and the

TABLE 1. THE EFFECT OF EXPOSURE TO COLD ON THE ASCORBIC ACID AND CHOLESTEROL CONTENT OF THE ADRENAL GLAND OF NORMAL AND DIABETIC RATS

Group	No. of Rats	Treatment	Adrenal Ascorbic Acid Mean and Standard Error (mg./100 gm.)	Adrenal Cholesterol Mean and Standard Error (mg./100 mg.)	Blood Sugar mg.%	
					Min.	Max.
1	13	None	356 ± 11.8			
2	10	Diabetic* Room temperature	359 ± 12.4		235	450
3	10	Normal 1 hr. 5–7° C.	257 ± 11.7		86	130
4	12	Diabetic 1 hr. 5–7° C.	273 ± 15.2		252	412
5	19	None		3.86 ± 0.18	97	120
6	14	Fasted 24 hrs.		3.82 ± 0.19	90	130
7	16	Diabetic		4.32 ± 0.06	160	500
8	7	Normal non-fasted 4 hrs. at 3–5° C.		3.61 ± 0.28	97	125
9	18	Normal fasted 24 hrs. 4 hrs. at 3–5° C.		2.63 ± 0.22	78	132
10	9	Diabetic 4 hrs. at 3–5° C.		2.50 ± 0.35	190	344

* Diabetic animals received 55 mg. per kilogram of alloxan, intravenously, 7 to 9 days before the determinations were done.

standard error of the mean are recorded in each instance. The mean values, 356 ± 11.8 mg. per 100 gm. for normal rats, and 359 ± 12.4 mg. per 100 gm. for diabetic rats indicate that diabetes does not affect the adrenal ascorbic acid level of non-stressed animals. Following one hour of exposure to cold (groups 3 and 4, Table 1) the ascorbic acid values were 257 ± 11.7 for the normal rats and 273 ± 15.2 for the diabetics, a

difference which is not significant ($P > 0.4$).[2] These values indicate that the adrenal of the diabetic maintains a normal level of ascorbic acid which is able to respond to cold exposure in a manner comparable to that of the normal animal.

Groups 5, 6, 7, Table 1, compare the adrenal cholesterol values of non-stressed animals which were either normal and not fasted (group 5), normal and fasted for 24 hours (group 6) or diabetic and not fasted (group 7). The mean values of adrenal cholesterol for groups 5 and 6, 3.86 ± 0.18 mg. and 3.82 ± 0.19 mg. per 100 mg., respectively, are practically identical. Group 7, the diabetic group, showed a mean of $4.32 \pm .06$ mg. per 100 mg. This is an increase of 11.0 per cent over the mean of group 7, the comparable non-diabetic group, but the increase is of doubtful significance ($P > 0.05$). Group 8 shows the effect of exposure to cold for four hours on the adrenal cholesterol of the normal, non-fasted rat, and group 9 shows the results of the same treatment on rats fasted for 24 hours prior to the exposure. The group which had not been fasted showed little effect of the cold stress (6 per cent decrease of cholesterol) whereas the fasted group showed a decrease of 31 per cent. Statistical comparison of the two means shows $P < 0.01$.

In contrast to the above findings, when non-fasted diabetic animals were subjected to cold (group 10) their adrenal cholesterol values decreased 42 per cent as compared to their unstressed controls (group 7). This response is similar to that seen in the normal, fasted group rather than the normal, non-fasted group.

DISCUSSION

Few reports are available concerning the effect of diabetes on the function of the adrenal cortex. In addition to the work cited above Pincus (1949) reported briefly that there is evidence for increased adrenal activity in alloxan diabetes and that adrenal cholesterol was very low. This is in contrast to the results reported herein, but a direct comparison is impossible pending a detailed report of the experiments of Pincus, Scola and Elmadjian (Ref. 14, p. 64 of the Macy Adrenal Cortical Conference, 1949). There are so many possible degrees of severity of diabetes that their findings may have been obtained on experimental animals whose metabolic state varied widely from those herein reported.

The usual technique for the determination of stress responses employs a period of fasting preliminary to the application of stress. The fact that adrenal cholesterol responds to cold by decreasing markedly in fasted normal and non-fasted diabetic rats, but that the decrease is much less

[2] Probability by "Student" method of *t*. Binnett, L. C. H.: Methods of Statistical Analysis, Williams and Norgate, Ltd.: 3rd ed., p. 114, 1941.

in non-fasted normal rats, seems to have one possible explanation which rests upon a common mechanism. Readily available stores of carbohydrate as liver glycogen would be depleted in the fasted animal, whereas the utilization of glucose would be curtailed in the diabetic. In either case a compensating mechanism consists of the transformation of protein to carbohydrate, a reaction controlled in part by adrenal cortical hormones (Long, Katzin and Fry, 1940; Engel, 1951). Both of the groups which responded to the cold environment (Groups 9 and 10, Table 1) were in a condition in which either inadequate supplies of carbohydrate or inadequate utilization would be expected, and the response to the stimulus might require gluconeogenesis.

Such a demand for carbohydrate from protein would also involve participation of adrenal cortical hormones (Engel, 1951). On the assumption that the use of these hormones in the tissues would decrease their total quantity and circulating blood level (Sayers, 1950), the anterior pituitary would then have been permitted greater output of ACTH with a resultant decrease of adrenal cholesterol.

A fundamental relationship between glucose and the action of adrenal cortical extract on protein metabolism has been elucidated by Engel and coworkers (Engel, Schiller and Pentz, 1949; Bondy, Engel and Farrar, 1949; Engel, 1949; Bondy, 1949). That this relationship could extend to the regulation of the secretion of adrenal cortical hormones seems a reasonable possibility.

Constantinides (1950) has reported minimal adrenal responses to stress in animals fed high carbohydrate diets as compared to animals fed isocaloric, high amino acid diets. In a later paper, however, Constantinides, Fortier and Skelton (1950) reported that glucose administration augmented the response of adrenal cholesterol to ACTH in hypophysectomized animals. Elmadjian, Freeman and Pincus (1946) also reported activation of the adrenal cortex by glucose in the intact animal, a finding similar to that of Jordan, et al. (1950). These apparent divergencies in the reported relation of glucose to adrenal cortical activity need not necessarily be incompatible, but the effect of hyperglycemia as an adrenal stress in the intact animal and the possible role of carbohydrate stores in regulating adrenal function require reconciliation.

Consideration of the adrenal ascorbic acid changes, Table 1, reveals that fasting was unnecessary to insure reduction of ascorbic acid in the cold environment. Sayers (1950) recognizes that ascorbic acid changes and cholesterol changes in the adrenal gland occasioned by stress may not be absolutely related phenomena. Several reports indicate this possibility (McKee, Cobbey and Geiman, 1949; Murray, 1948; Doughaday, Jaffe and Williams, 1948; Long, 1947). The results herein reported may

be further indication of an incomplete relationship between the glandular metabolic roles of these two substances.

The adrenal weights of the female rats showed a definite increase as the result of diabetes, but this was not duplicated in the males. Consequently, no definite relation of cause and effect in this situation can be stated at this time.

SUMMARY

The response of the adrenal glands of normal and diabetic rats following exposure to cold was determined by measurement of adrenal cholesterol and ascorbic acid content. Exposure to cold, without preliminary fasting, produced a marked decrease in adrenal cholesterol in the diabetic animals. A comparable decrease was not produced in normal rats by cold unless they had been fasted previously. Cold exposure caused a decrease of adrenal ascorbic acid in both normal and diabetic rats without preliminary fasting. These results suggest that the response of the adrenal gland to cold, as measured by a decrease of cholesterol concentration, is related to curtailment of metabolically available carbohydrate.

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A QUANTITATIVE METHOD FOR THE BIOASSAY OF THE EFFECT OF ADRENAL CORTICAL STEROIDS ON MINERAL METABOLISM

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with the technical assistance of M. WOODFORD AND S. GRAVES

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THE importance of the adrenal cortex in the regulation of mineral metabolism has been recognized since 1932, and much work has been carried out on the disturbances which result after adrenalectomy and the restoration to normal conditions following therapy with adrenal cortical extracts (Bauman & Kurland 1927, Loeb 1932, see also reviews by Gaunt 1949, Kendal 1948 and Noble 1950). Despite this, no bioassay method based on the direct measurement of this effect, has been developed to date comparable as regards accuracy and sensitivity with the liver glycogen test for the action of substances on carbohydrate metabolism (Venning 1946, Dorfman 1946 and Eggleston 1946).

Mineral activity may be assessed by measuring the alteration of sodium and potassium levels either in plasma (Hartman & Spoor 1940, Wirz 1950) or urine (Harrop 1936, Thorn et al. 1938). Both these measurements are subject to wide variation arising from alterations in external factors such as diet, but estimations in urine have the advantage of greater sensitivity as well as ease of technique. The index of mineral activity may be based on the estimation of either inert or radioactive elements. The development of the flame photometer has made possible the accurate and rapid estimation of small quantities of sodium and potassium in urine or plasma. This has been used by two independent workers for the quantitative determination of the mineral corticoid activity based on retention of inert sodium. Deming and Luetscher (1950), using adrenalectomized rats, compared sodium excretion during a control and test period of 5 hours on successive days and were able to demonstrate the sodium retaining effect of about 10 μg. DOC or DOCA. They have attempted to reduce variation and increase urine output by the injection of a water load shortly before the start of the test. However, their results showed wide variation and no dose response curve was given. Spencer (1950) obtained more satisfactory results using 35 gm. adrenalectomized mice; he has shown that sodium retention was linearly proportional to the logarithm

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of the dose over a range of 0.5 to 4 μg. DOCA. The accurate injection of a constant high sodium load using an all glass micrometer syringe enabled Spencer to reduce the variation. This method had the disadvantage of being very time consuming and required a preliminary test for the selection of suitable experimental animals followed by a cross-over test of 6 hours on two or three successive days. Moreover Dorfman et al. (1947) have shown that sensitivity was greatly reduced by injection of a high sodium load. Dorfman (1947 & 1949) has used radio sodium to measure the retention of the injected dose and radiopotassium to measure potassium loss as a basis for separate bioassay methods. In both cases, with adult adrenalectomized rats as the test animals, the sensitivity was about 10 μg. DOCA but the results were intended to be qualitative only and no dose response curve has as yet been published.

A method using radioactive isotopes appears to have certain advantages over measurement of inert sodium necessitating the injection of a high sodium or water load. By employing tracer doses the test is made to approach a true balance experiment carried out under physiological conditions. The technique to be described below was an extension of Dorfman's method whereby Na^{24} and K^{42}, instead of being used separately, were injected simultaneously in a solution containing a very low sodium and comparatively high potassium load, the total mineral load, however, falling far below any used for inert measurements. The two isotopes were measured in the urine by a rapid physical method of analysis and the activity of the test substance was assessed by its influence on the Na^{24}/K^{42} urinary ratio.

PRELIMINARY EXPERIMENTS

In preliminary experiments, the injection of DOCA and radioactive sodium and potassium into 140 gm. adrenalectomized rats was immediately followed by the collection of urine for six hours. The urinary Na^{24}/K^{42} ratio was found to be linearly dependent on the logarithm of the dose of DOCA for a range of 4–13 μg. It was decided to reduce the weight of the rats to about 30 gm. and the time of collection of urine to 2 hours in order to increase the sensitivity of the assay. By so doing, it was also hoped to include the effect of those corticoids whose action on mineral metabolism is of short duration (Wirz 1951), as well as to reduce the total time taken for the bioassay. However it was found that inconsistent readings resulted from the use of a short period of collection presumably due to the slow rate of absorption of DOCA from the solvent, arachis oil. Subsequent investigations showed that a suitable solvent (a) must itself be non-toxic and have no effect on the urinary Na^{24}/K^{42} ratio, (b) must be a good solvent for the test substance, (c) must be of such a nature that the test substance

is rapidly absorbed into the blood after subcutaneous injection of the solution. Of the solvents commonly used for adrenal steroids only ethyl alcohol was non-toxic to experimental animals and even this, unless used in very small quantities, had an effect on the Na^{24}/K^{42} ratio. A suitable compromise was found in aqueous 20% ethyl alcohol which had no effect on the radioactive ratio when injected in quantities less than 0.2 ml. into 30 gm. rats. The adrenal cortical hormones were sufficiently soluble in 20% ethyl alcohol to permit the injection of 10–20 μg. in 0.1 ml. This was a suitable quantity as the range and sensitivity of the assay was now found to be from 0.5–5 μg. DOCA. The procedure described in detail below was therefore adopted in subsequent investigations.

METHOD

The animals used throughout these experiments were 3–4 week old male Wistar rats, 30–40 gm. in weight, bred in this Institute. Bilateral adrenalectomy was performed by the dorsal route under ether anaesthesia. After operation the rats were fed the stock diet of rat cubes (3.8 mg. sodium and 7.2 mg. potassium per gm. original matter) but drinking water was replaced by a solution of 1% saline and 5% glucose. The assay was carried out 4 days after operation, during which time, temperature and humidity were kept constant. Animals which showed an abnormally high gain in weight or were very weak on the day of the assay were omitted from the test. The presence of any residual cortical tissue was checked by observing the survival time following the withdrawal of saline at the end of the assay. Completeness of adrenalectomy was indicated by a survival time of less than 8 days.

At 11 A.M. on the day of the assay, the rats were injected subcutaneously with a solution of the hormone in 0.1 ml. 20% alcohol or with the aqueous alcohol only in the case of the controls. This was followed one hour later by a subcutaneous injection of 0.5 ml. of an aqueous solution of sodium and potassium chloride labelled with Na^{24} and K^{42}, which was prepared by dissolving the products of neutron irradiation of sodium and potassium bicarbonate and titrating with hydrochloric acid to a pH value of 7.5. 0.5 ml. of injected solution prepared in this manner contained 27 μg. sodium and 381 μg. potassium and a total radioactivity of approximately 10 μ curies on the day of the assay. Immediately following the injection, each rat was placed into a 500 cc. beaker fitted with a wire mesh. At the end of the two hours collection period the rats were made to urinate by applying suprapubic pressure and giving a light whiff of ether. The beakers and wire meshes were then rinsed with a small volume of 70% alcohol and the contents poured into Petri dishes (4 cm. in diameter) and evaporated to dryness. The Petri dishes were placed under an end window Geiger counter and the counts per min. due to Na^{24} divided by the counts per min. due to K^{42} was determined for each sample, and for an aliquot of the injected solution by a physical absorption technique the details of which will be published elsewhere. The ratios for the samples were expressed as a fraction of the latter value.

It has been shown that there is a diurnal variation in the rate of sodium excretion (Simpson & Tait 1950). Thus, even if the time interval between injection of the solutions and collection of the urine was kept constant, systematic errors between groups might occur. The following procedure was therefore adopted in order to minimize this source of error, viz., in the usual balanced assay, 6 groups a, b, c, · · · f, containing 8 rats per

group, were injected with 6 doses of hormone in the following order: $a_1 \cdots f_1$, $a_2 \cdots f_2$, and so on to $a_8 \cdots f_8$. The beakers were arranged and urine collected in the same order to avoid any errors due to variation in temperature or humidity with position and to keep the time interval to collection constant. There was no need to correct for decay of the Na^{24}/K^{42} ratio when the samples were measured for radioactivity in this sequence.

RESULTS

Although desoxycorticosterone is not now considered to be present in adrenal cortical extracts, it is a synthetic compound in plentiful supply and has a potent action on mineral metabolism. For these reasons, preliminary investigations were carried out with this substance. 0.83, 1.25, 1.88 and 2.82 μg. DOCA[1] were injected in the manner previously described, using 8 rats per dose of steroid and 8 rats as controls. This procedure was repeated for two successive weeks; the results are presented in Graph I,

TABLE 1. STATISTICAL ANALYSIS OF EFFECTS OF A.C.E. AND DOCA ON ACTUAL Na^{24}/K^{42} RATIO OR RATIO EXPRESSED AS % OF CONTROLS

Dose response	P Value for block differences	Equation (Combined)	$b \pm S_b$	λ —index of precision $= S/b$
Actual ratio DOCA I & II	0.05	Actual ratio $Na^{24}/K^{42} = 1.531 - 1.647$ (log. DOCA in μg.—0.1848)	-1.613 ± 0.281	I 0.267 .....Comb. 0.267 II 0.295
Actual ratio ACE I & II	0.01	Actual ratio $Na^{24}/K^{42} = 1.500 - 1.476$ (log. ml. extract—2.047)	$-1,476 \pm 0.277$	I 0.251 .....Comb. 0.295 II 0.393
Ratio % controls DOCA I & II	>0.2	Ratio as % of controls $= 64.48 - 70.42$ (log. DOCA in μg.—0.1848)	-70.42 ± 11.30	0.252
Ratio % controls ACE I & II	>0.2	Ratio as % of controls $= 68.01 - 66.89$ (log. ml. extract—2.047)	-66.89 ± 13.28	0.313

Ratio (actual or % of controls) $= a + b(x - \bar{x})$.
S_b = standard error of b.
$S = \sqrt{\text{error mean square}}$

with the urinary Na^{24}/K^{42} ratios plotted against the logarithm of the dose DOCA. Analysis of variance of the results showed that there was a significant negative regression with dose ($P < 0.001$) and the linearity of the ratio to logarithm dose response curve was not in doubt. There was, however, a significant difference between the blocks of the two sets of results ($P = 0.05$). The index of precision (mean error square/slope) for the two results was 0.267 and 0.295.

Exactly the same procedure was repeated using adrenal cortical extract.[2] The doses were in the same geometrical progression with a minimum dose equivalent of 0.0049 ml. of original extract per rat. The results were very similar to those obtained for DOCA as can be seen in Graph II and Table 1. Again there was a significant negative linear regression of the

[1] The DOCA used throughout these experiments was supplied by Organon Ltd.

[2] Adrenal Cortical Extract, Eucortone, was supplied by Allen and Hanbury's Ltd.

ratio with logarithm dose of adrenal extract and a block difference between the two successive sets of results. The index of precision for the two sets was 0.251 and 0.393.

The mean index of precision of 0.30 showed that the use of 24 rats for unknown and standard substance would give fiducial limits of activity of about 66–151%. Whilst the index of precision is high compared with the carbohydrate methods of Venning (1946) and Eggleston (1946) and others, it promised to give the required information on mineral activity

TABLE II. THE POTENCY OF ADRENAL STERIODS IN TERMS OF DOCA (100) MEASURED BY THE LOWERING OF Na^{24}/K^{42} URINARY RATIO COMPARED WITH ACTIVITY AS MEASURED BY THE EVERSE DE FREMERY MUSCLE TEST AND BY THE SURVIVAL TIME METHOD OF CARTLAND AND KUIZENGA

	Compound	Na^{24}/K^{42} urinary ratio		Muscle test	Survival time
		Activity per unit mass	Exact Fiducial Limits P 0.05	Activity per unit mass	Activity per unit mass
DOC	11-desoxycorticosterone	135	93–209	—	—
	11-desoxycorticosterone acetate	100		100	100
Kendall's B	Corticosterone	14.3	9.1–21.1	7–9	17
Reichstein's S	17-OH-11 desoxycorticosterone acetate	7.99	5.1–11.0	7	—
Kendall's F	17-OH-corticosterone	7.58	3.3–14.9	3.5–4	3–6
Kendall's A	11-dehydrocorticosterone	—		—	10
	11-dehydrocorticosterone acetate	6.74	2.5–10.9	—	—
Kendall's E	17-OH-11-dehydrocorticosterone	5.91	3.8 –7.9	3.5	3–6
	17-OH-11-dehydrocorticosterone acetate	5.16	3.1 –7.7	—	—
	Progesterone	3		0	—
	Oestradiol	3		—	—
	Testosterone	1.5		—	—
	Adrenal Extract (Beef)	1 ml. =150.5 DOCA	118 –189	—	1 ml. =60–200 DOCA

In 6 point balanced assays, 0.83, 1.25, 1.88γ DOCA were used and three doses of steroid of approximate equivalent activity.

of various steroids. The differences between blocks showed that for quantitative assays, simultaneous use of standard and unknown was essential. This presents no difficulty if DOCA is used as the standard.

However, the optimum employment of such balanced assay methods requires the comparison of quantities of compounds of approximately equal activity. It was therefore considered useful to have a standard curve which was applicable from week to week for obtaining the approximate specific activity of compounds before carrying out the more quantitative assay. An examination of the previous results was undertaken with this object.

Graphs I and II showed that the block totals of Na^{24}/K^{42} might be

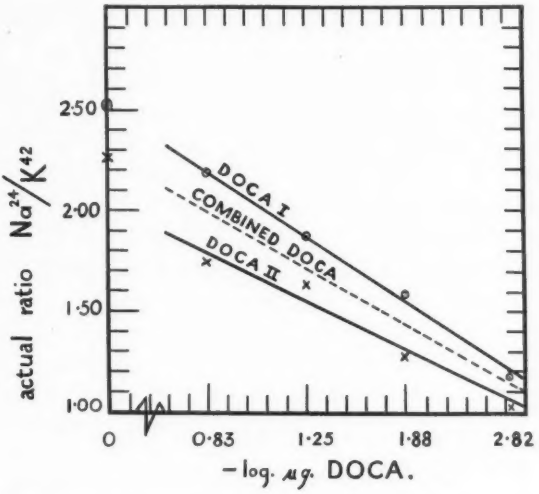

FIG. 1. The effect of 11-desoxycorticosterone acetate (DOCA) on the urinary Na^{24}/K^{42} ratio on two separate weeks.
⊙ Na^{24}/K^{42} ratio of controls week I
X Na^{24}/K^{42} ratio of controls week II

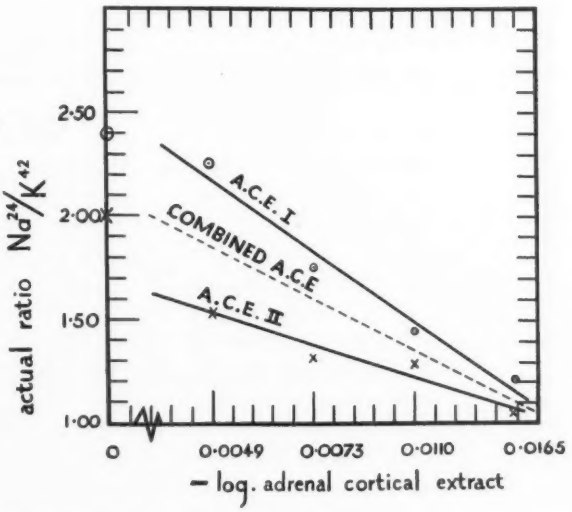

FIG. 2. The effect of adrenal cortical extract (ACE) on the urinary Na^{24}/K^{42} ratio on two separate weeks.
⊙ Na^{24}/K^{42} ratio of controls week I
X Na^{24}/K^{42} ratio of controls week II

correlated with the corresponding control values. Expression of the ratios as % of the control values in any one set, resulted in a disappearance of the differences in blocks and slopes as is indicated in Table 1. Graph III shows a comparison of the combined slopes of DOCA and A.C.E., all ratios being expressed as % of the appropriate control values. The results have been extended to 9 μg. DOCA. The graph shows, and statistical analysis confirms, that the slopes for A.C.E. (between 0.0049 ml and 0.0165 ml) and DOCA (between 0.83 μg. and 4.23 μg.) are linear and parallel.

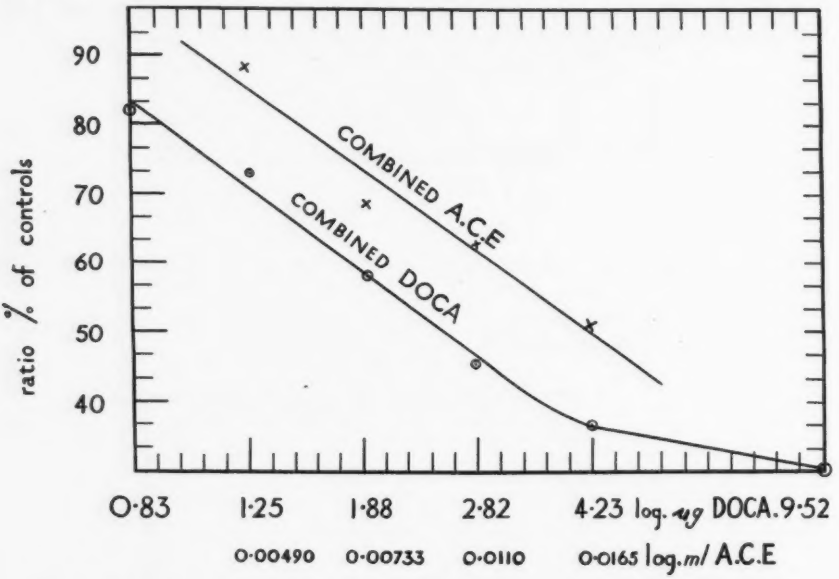

FIG. 3. The effect of adrenal cortical extract (ACE) and 11-desoxycorticosterone acetate (DOCA) on the urinary Na^{24}/K^{42} ratio.
The results of two weeks assays were pooled and expressed as % of the respective Na^{24}/K^{42} control ratios.

Stringent statistical justification for the procedure of constructing such a standard curve would involve an analysis of covariance of several sets of results. As the procedure was desired as a preliminary test only, and subsequently gave reliable results in practice, this was not done. However examination of the urinary Na^{24}/K^{42} ratios obtained on injecting 1.88 μg DOCA or alcohol only into groups of rats on eight subsequent occasions showed that those obtained for the rats injected with steroid exhibited a significant linear regression with concomitant control mean values, the curve having a very high probability ($P>0.5$) of passing through the origin.

Various steroids were now tested for approximate activity in this way and their potency was then compared with DOCA using a 6 or 4 point balanced assay, 24 rats per compound. The doses were graded in geometrical progression of 1.5 and occasionally 2.0. The main practical difficulty was the limited solubility of some of the compounds in 20% alcohol and because of this a light suspension of some of the acetates had to be used.

Desoxycorticosterone,[3] desoxycorticosterone acetate, corticosterone, 11-desoxy-17-hydroxycorticosterone acetate, 17-hydroxy-corticosterone, 11-dehydrocorticosterone acetate, and 11-dehydro-17-hydroxycorticosterone acetate were found to be active in descending order of potency per weight. Oestradiol, progesterone and testosterone were found to be inactive in the doses used and no compound was found which raised the Na^{24}/K^{42} ratio. The potency ratios with exact fiducial limits are presented in Table II. For all compounds tested, the linearity and parallelism of the slopes compared with DOCA were not in doubt and hence the calculation of a potency ratio was justified. The determination of the exact slope for these compounds would, however, involve the use of large numbers of animals which has not yet been undertaken.

DISCUSSION

The aim of this work was to develop a method for the quantitative measurement of the ability of substances to restore the normal electrolyte balance in adrenalectomized rats. The application of such a method in the isolation of active principles in cortical extracts and in the testing of synthetic compounds would be most valuable at the present time.

The predominant action of corticoids in prolonging the life of adrenalectomized rats maintained on a normal salt diet and under low stress conditions is undoubtedly one on mineral metabolism. This has been shown by the great increase in survival time produced by salt therapy alone and the failure of glucose to produce the same results (Loeb, 1933; Rubin and Krick, 1936; Marine and Baumann, 1927; Corey, 1927). The restoration of potassium as well as sodium levels in the plasma has been shown by Harrison and Darrow (1939) to be the result of salt therapy. The method of Cartland and Kuizenga (1936) based on survival time measures such an effect but as a practical bioassay method has serious disadvantages. The test is of necessity of long duration and requires large quantities of test material. The quantal nature of the response makes statistical

[3] We are grateful to Professor T. Reichstein for the gift of corticosterone, 11-dehydrocorticosterone acetate, 17-OH-11-desoxycorticosterone acetate; to Ciba Laboratories Ltd. for 11-desoxycorticosterone and 17-OH-11-desoxycorticosterone acetate and the Medical Research Council for cortisone acetate.

analysis of the results more difficult, moreover the animals must be guarded against all stress conditions if false values are to be avoided. A more fundamental objection is that certain experiments have shown that the effect on electrolyte metabolism is not the only factor in survival after adrenalectomy (Swingle et al., 1936, 1938). Thus it would appear that a more direct bioassay method of the effect on electrolyte balance is essential.

The action of cortical steroids on mineral metabolism is of a very complex nature and includes the regulation of body compartment concentrations as well as effects on renal function such as on glomerular filtration rate and proximal and distal tubular reabsorption. By selection of suitable experimental conditions it may be possible to base a bioassay method on any of these particular effects on any mineral element, but it seems logical to restrict an investigation to the effects on sodium and potassium metabolism as these electrolytes play the dominant part. From the point of view of both sensitivity and ease of technique this measurement is best undertaken in urine.

The measurement of the urinary Na^{24}/K^{42} ratio after the injection of hormones and radioactive isotopes would seem to provide such an assessment of electrolyte balance. In addition the technique reduces errors in the following manner:—

(i) errors in the volume of injected solution of the isotopes produces variation in the urinary ratio only in so far as the load varies.

(ii) errors in the preparation of the sample caused by sputtering are eliminated and decay corrections can be neglected when measuring the Na^{24}/K^{42} ratio.

(iii) incomplete collection of urine which can cause considerable errors when sodium or potassium only are measured results in variation only in so far as the urine sample may not be representative of the complete collection time.

The reduction of errors in the collection of urine makes possible much shorter times of collection than has been used in other methods. This is of advantage in decreasing the total time of the assay and in enabling the measurement of the effect of those hormones, such as 17-OH-11-dehydrocorticosterone, whose action on mineral metabolism has been shown to be of short duration only (Wirz, 1951). The authors have been able to detect the effect of cortisone on the Na^{24}/K^{42} urinary ratio in a two hour collection time but not in 6 hours, whereas DOCA is effective in both cases. However, as previously discussed, the use of short times of collection demands the use of a solvent of suitable characteristics.

Table II shows the potencies per unit weight of various steroids in terms of DOCA as measured by the method described here. These values, with

their exact fiducial limits (P 0.05), are compared with the potencies as measured by the survival time method of Kuizenga and Cartland (1939, 1940) and the Everse de Fremery test (1939, 1943) which is based on the recovery of muscle fatigue in the adrenalectomized rats. The comparison shows qualitative and quantitative agreement between the three methods. Thus, all the corticosteroids act on the ratio in the same direction corresponding with the unidirectional action of the compounds in the Kuizenga and Everse de Fremery tests.[4] The use of acetates in some tests and the corresponding free compounds in others tends to obscure the quantitative aspects of the comparison, nevertheless it is clear that there is close quantitative agreement between the tests. If the depression of the Na^{24}/K^{42} urinary ratio is taken as a true index of mineral activity, this confirms that the Kuizenga and Everse de Fremery tests specifically measure the action of compounds on electrolyte metabolism, which has been assumed in the past on indirect evidence only.

The activity of adrenal cortical extract in 20% alcohol, as measured by this test, is extremely high compared with the activities of saturated solutions of the known corticosteroids in the same solvent. It is even twice as great as a saturated solution of DOC, which in any case we have been unable to detect by paper chromatographic methods in this particular extract. Investigation of this finding is continuing.

The main advantages of this method can be summarized as follows:—

(a) great sensitivity; 0.4 μg 11-desoxycorticosterone can be detected.
(b) qualitative and quantitative correspondence with the survival time method of Cartland and Kuizenga and the Everse de Fremery muscle test.
(c) ease of technique.
(d) rapidity of total assay (total time including measurements about 6 hours)
(e) parallelism of slopes in balanced assays
(f) the inclusion of corticosteroids with short duration of action.

The main disadvantages are:—

(a) the test animals can be used for one assay only
(b) solvent is not entirely suitable and probably causes some variation
(c) high index of precision compared with the liver glycogen deposition methods of Eggleston, Venning and Dorfman.

[4] Dorfman found increased excretion of Na^{24} after injection of 17-hydroxycorticosterone in contrast to retention as for DOCA. Using cortisone acetate which behaves in a similar manner to 17-hydroxycorticosterone we have confirmed this finding under similar load conditions but found no effect on the urinary Na^{24}/K^{42} ratio (unpublished work). On lowering the sodium load and decreasing the time of collection of urine the ratio was decreased as described in the text.

SUMMARY

A method has been developed for the bioassay of the effects of corticosteroids on mineral metabolism based on the depression of the urinary Na^{24}/K^{42} ratio in adrenalectomized rats. The lowering of the ratio was linearly dependent on the logarithm of a dose of DOCA for a range of 0.8–4.0 μg. The slopes of the ratios against logarithm of dose for adrenal cortical extract and the active cortical steroids were found to be linear and parallel with that of DOCA. Block variation from test to test necessitated the use of 6 point balanced assays with DOCA as standard. A standard curve could be constructed for the approximate assay of substances by expressing the ratios of the compounds tested as % of concurrent control values as block variation was eliminated by this procedure.

All the active compounds tested lowered the urinary Na^{24}/K^{42} ratio and the activities in terms of DOCA showed qualitative and quantitative agreement with the Kuizenga survival time test and the Everse de Fremery muscle test. Progesterone, oestradiol and testosterone were found to be inactive. Beef adrenal cortical extract was found to be highly active to an extent which is as yet unexplained.

The main advantages and disadvantages of the test are discussed.

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EFFECTS OF PURIFIED GONADOTROPHINS ON THE CHOLESTEROL CONTENT OF THE TESTES OF IMMATURE RATS[1]

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IN A recent study (Tepperman and Tepperman, 1947) it was found that the treatment of immature rats with a commercial gonadotrophin of pituitary origin (Gonadophysin) resulted in an accumulation of cholesterol ester in the testis. Chorionic gonadotrophin (CG) had no such effect, but, in animals primed with Gonadophysin, treatment with chorionic gonadotrophin resulted in a diminution in testis cholesterol ester associated with a marked enhancement of androgen output. Since Gonadophysin produced a marked increase in testis weight and comparatively small changes in seminal vesical weight, it was regarded as predominantly FSH. Chorionic gonadotrophin powerfully stimulated androgen production and the authors, in common with other workers (for example, see Burrows, 1949) believed that the terms "chlorionic gonadotrophin," "LH," and "ICSH" were virtually interchangeable designations in the male animal. The design of the studies reported in 1947, then, was based on these evaluations of the only preparations available to the authors when these experiments were done.

The following statement may be recalled at this time:

"Whether or not these changes are of physiologic significance cannot be stated until this work is repeated with purified FSH and ICSH preparations of hypophysial origin since, clearly, one cannot attribute such significance to changes obtained in the male with a chorionic gonadotrophin and a mixture of sheep pituitary gonadotrophins. In any case it is suggested that the changes described are consistent with the hypothesis that the gonadotrophins affect androgen production by virtue of their direct involvement in the metabolism of esterified cholesterol in the testis. A description of the precise nature of this involvement must await further work. One can only suggest that some constituent of Gonadophysin, possibly FSH, may increase the ester cholesterol content of the gland, either by accelerating the rate of local esterification or by increasing the rate of abstrac-

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tion of pre-esterified cholesterol from the circulating blood. ICSH may function in the conversion of esterified cholesterol into androgen and, possibly, in the release of androgen into the blood stream."

The availability of purified gonadotrophins of pituitary origin made it possible to reinvestigate this problem. The present report is concerned with the experimental demonstration of three facts: (1) purified FSH does not cause a significant change in testis cholesterol ester (2) purified LH from two different sources consistently caused a highly significant and readily measurable increase in testis cholesterol ester, and (3) since chorionic gonadotrophin by itself has no significant effect on testis cholesterol ester these data constitute evidence that pituitary LH and chorionic gonadotrophin have readily distinguishable effects in the immature male rat.

MATERIALS AND METHODS

Twenty-one day old intact rats (Albino Farms) were used in this study. They were maintained on a diet of Purina dog chow fed *ad libitum* and they had free access to water.

The following preparations were used:

(1) *FSH 150HM3*, kindly supplied by Drs. R. K. Meyer and W. H. McShan, who also placed their assay data at the disposal of the authors. One gram of dried, de-fatted sheep pituitary powder is equivalent to 5.6 mg. of purified FSH. When assayed in intact 21 day old female rats at a dose level equivalent to 2 gms. of starting material the ovarian weight was 66 mg. as compared with 22 mg. for control animals. None of the ovaries contained corpora lutea on gross examination.

(2) *LH 147HM3*, also assayed and supplied by Drs. McShan and Meyer. 16.4 mg. of this preparation is equivalent to 1 gm. of starting material. 0.5 gm. equivalent of this preparation, when combined with 0.5 gm. equivalent of FSH 130HM2, resulted in ovarian weights of 86 mg. and the ovaries contained numerous corpora lutea.

(3) *LH FW-234*, kindly supplied by Dr. John R. Mote, Armour and Company. On immature rat assay this preparation contains 159 Collip units per milligram. In hypophysectomized female rats a dose of 40 micrograms per day for four days causes a 100 per cent increase in ovarian weight and no increase in uterine weight. The preparation contains very small traces of prolactin, thyrothrophin, growth hormone, ACTH and pressor and oxytocic activities of the posterior lobe.

(4) *"FSH Synergist,"* generously furnished by the Schering Company. In spite of its name this material, presumably of sheep pituitary origin, has been found to possess definite LH activity by Jones (1948) and by Lloyd (1949).

(5) *PMS, Schering.*

(6) *Prolactin, Schering;* lot #220 pr 4, which contain 10 I.U. per milligram.

(7) *Follutein, Squibb*, a chorionic gonadotrophin for which we are indebted to Dr. R. Bates.

(8) *A.P.L.* (*Ayerst, McKenna and Harrison*), another chorionic gonadotrophin, which was kindly contributed by Dr. E. C. Reifenstein, Jr.

(9) *Gonadophysin, Searle*, a mixture of sheep pituitary gonadotrophins kindly donated by Dr. I. C. Winter of the Searle Company. The hormones were administered twice daily in divided doses by the subcutaneous route. The doses used are presented in connection with individual experiments.

Cholesterol analyses were performed according to the Sperry-Schoenheimer (1942) method. Digitonin precipitation was performed with aqueous digitonin. Seminal vesicles were emptied, blotted on filter paper and weighed on the micro-torsion balance by the same observer in all experiments reported. Means and their standard errors are presented in all tables. The criterion for statistical significance throughout is a probability level of less than 0.01 calculated according to student's "t" test (Fisher, 1941).

RESULTS

The experimental results are summarized in a series of five tables.

Examination of Table 1 reveals that treatment of immature male rats with a purified preparation of FSH causes no increase in testis cholesterol ester. This is true in spite of the fact that the FSH may have been con-

TABLE I. EFFECT OF PITUITARY FSH AND LH (McSHAN AND MEYER) ON TESTIS CHOLESTEROL CONTENT

The dose of FSH was 1.5 mg. per rat twice daily for 6 days; of LH 3 mg. per rat twice daily for 6 days. Means and standard errors for groups of 6 rats are given. In this and subsequent tables, figures significantly different from control values are printed in bold-faced type ($P < 0.01$).

Treatment	Final body weight gm.	Testis wt. mg./100 gm. of rat	Seminal vesicle wt. mg./100 gm. of rat	Total testis cholesterol mg./100 gm. of rat	Ester cholesterol mg./gm. testis (wet wt.)	Ester cholesterol of testis mg./100 gm.
Saline	56 ± 5.3	784 ± 41.6	16.6 ± 0.9	1.72 ± .059	.02 ± .014	.02 ± .014
FSH	55 ± 4.2	**952 ± 21.3**	**21.4 ± 1.1**	**1.96 ± .045**	.06 ± .027	.05 ± .024
LH	53 ± 2.1	**1046 ± 41.8**	**49.7 ± 5.6**	**2.36 ± .091**	**.28 ± .047**	**.27 ± .041**

taminated with small amounts of LH, since there was a small but statistically significant increase in seminal vesicle weight. In contrast, the same table shows that treatment with a purified LH preparation causes a comparatively large increase in both concentration and absolute amount of cholesterol ester in the testis. This increase is associated with evidence of markedly enhanced androgen secretion as indicated by a threefold increase in seminal vesicle weight. Examination of microscopic sections of these glands by Dr. Herman Dick revealed striking accumulations of sudanophilic lipid in the interstitial cells of LH-treated rats as compared with similar cells of control animals and FSH-treated ones.

In order to inhibit the production and release of endogenous gonadotrophins (Nalbandov and Baum, 1948), weanling male rats were implanted with stilbestrol pellets 25 days before gonadotrophin injections were begun. Groups of seven rats were injected with purified FSH, purified LH, and a combination of the two. The results are given in Table 2. Again, it is apparent that FSH produces no significant increase in absolute amount of cholesterol, while LH treatment and combined treatment with both hormones yielded results similar in direction to those seen in the first

TABLE II. EFFECTS OF PURIFIED GONADOTROPHINS IN STILBESTROL PELLET-IMPLANTED RATS

The doses used were 1.5 mg. FSH (McShan and Meyer) twice daily for 7 days and 3 mg. LH (McShan and Meyer) twice daily for 7 days. A stilbestrol pellet (15 mg.) was implanted in each rat at weaning 25 days before injections were started. Means and standard errors for groups of 7 rats are given.

Treatment	Final body weight gm.	Testis wt. mg./100 gm. of rat	Total testis cholesterol mg./00 gm. of rat	Ester cholesterol mg./gm. testis (wet wt.)	Ester cholesterol of testis mg./100 gm. of rat
Saline	116 ± 3.0	149 ± 6.1	.45 ± .023	.51 ± .08	.08 ± .014
FSH	116 ± 3.1	**322 ± 23.3**	**.76 ± .058**	.33 ± .03	.11 ± .009
LH	119 ± 4.7	**444 ± 24.4**	**.95 ± .046**	.40 ± .04	**.17 ± .010**
FSH + LH	118 ± 3.5	**575 ± 33.6**	**1.02 ± .057**	.26 ± .04*	**.15 ± .020**

* $P < 0.02$.

experiment. Here, fluctuations in gland size, doubtless caused primarily by changes in tubular mass, make interpretations of changes in cholesterol ester concentrations difficult. In this experiment the effect of stilbestrol on the seminal vesicles made it impossible to use the weight of these structures as an index of androgen secretion in response to the gonadotrophins.

In a previous experiment (Tepperman and Tepperman, 1947) it was shown that treatment with Gonadophysin caused an increase in testis cholesterol ester and subsequent treatment with chorionic gonadotrophin produced a decrease in cholesterol ester in association with a marked augmentation of androgen output. Following the demonstration that the administration of several preparations of purified pituitary LH resulted in

TABLE III. EFFECTS OF CHORIONIC GONADOTROPHIN (CG) ON THE TESTIS CHOLESTEROL CONTENT IN RATS PRE-TREATED WITH PITUITARY LH (ARMOUR)

The dose of LH was 5 units twice daily for 6 days. CG was given to pre-treated rats in a dose of 20 units twice daily for 4 days. Means and standard errors for groups of 10 rats are given.

Group	Treatment	Final body weight gm.	Testis wt. mg./100 gm. of rat	Seminal vesicle wt. mg./100 gm. of rat	Total testis cholesterol mg./100 gm. of rat	Ester cholesterol mg./gm. testis (wet wt.)	Ester cholesterol of testis mg./100 gm. of rat
A	Saline 6 days	65 ± 3.0	743 ± 46.4	16.7 ± 0.96	1.57 ± 0.10	0.06 ± 0.02	0.05 ± 0.01
B	LH 6 days	62 ± 1.5	859 ± 41.6	**32.8 ± 2.68**	**1.99 ± 0.06**	**0.30 ± 0.03**	**0.27 ± 0.02**
C	Saline 6 days CG 4 days	78 ± 3.5*	**1073 ± 33.2**	**75.0 ± 3.51**	1.90 ± 0.04	0.02 ± 0.08	0.02 ± 0.01
D	LH 6 days CG 4 days	76 ± 2.8*	**1055 ± 35.8**	**78.2 ± 3.52**	1.88 ± 0.07*	0.05 ± 0.02	0.05 ± 0.03
E	LH 6 days Saline 4 days	**82 ± 2.2**	**977 ± 34.2**	19.1 ± 0.94	1.85 ± 0.05	0.11 ± 0.02	0.10 ± 0.02

* $P < 0.02$.

an apparent piling up of esterified cholesterol in the testis, it seemed desirable to test the effect of chorionic gonadotrophin on LH-primed glands. Accordingly, the experiment described in Table 3 was done. Here, groups of animals were injected with purified LH and the increase in testis cholesterol ester was confirmed. Some of these animals were then injected with chorionic gonadotrophin for 4 days, while others were injected with physiological saline. Another group was given saline instead of LH, and subsequently injected with chorionic gonadotrophin. In the original Gonadophysin experiment there was no difference in the testis cholesterol ester content between the 6 day Gonadophysin group and the 6 day Gonadophysin-4 day saline group. In the present experiment, however, treatment of LH-primed rats with saline for 4 days was associated with a marked fall in both cholesterol concentration and absolute amount. There was a trend toward a further fall in the chorionic gonadotrophin group but the amounts of the material measured were small and the differences are not regarded as statistically significant. It is noteworthy that the saline-CG group (C) tended to be lower than the saline control group (A). Further, in another experiment, in which LH and CG were given simultaneously, there is some suggestion that the CG inhibited the rise in cholesterol ester seen after LH alone. While none of these experiments alone confirms our previous finding in the Gonadophysin experiment unequivocally, all of them viewed together suggest that CG causes a diminution in testis cholesterol ester in association with an increase in androgen output while LH of pituitary origin causes a piling up of cholesterol ester while stimulating androgen production. In other words, while these two substances may stimulate androgen production in common, they are readily distinguishable from one another in their effects on the chemical morphology of the testis.

In 1947 Everett clearly demonstrated that treatment of rats with pituitary LH increases the amount of cholesterol in the corpus luteum. When LH pre-treated rats were injected with luteotrophin a disappearance of cholesterol from the gland occurred, presumably associated with an increased rate of liberation of progesterone. The fact that luteotrophin, or prolactin, can be extracted from the pituitaries of male animals may or may not signify that this material has a physiologic function in the male. In any case, since it was possible to demonstrate an increase in the cholesterol ester content of the testis with the same type of LH preparation employed by Everett in his studies on the corpus luteum, a study of the effect of prolactin on LH pre-treated rats appeared to be warranted. Accordingly, an experiment similar to the one described previously with CG was undertaken. The results of this experiment are given in Table 4.

It is immediately apparent that LH treatment for 6 days markedly

TABLE IV. EFFECT OF PROLACTIN (SCHERING) ON THE TESTIS CHOLESTEROL CONTENT IN RATS PRE-TREATED WITH PITUITARY LH (ARMOUR)

The dose of LH was 5 units twice daily for 6 days. Prolactin was given to pre-treated rats in dose of 5 units twice daily for 4 days. Means and standard errors are given.

No. of rats	Treatment	Final body wt. gm.	Testis wt. mg./100 gm. of rat	Seminal vesicle wt. mg./100 gm. of rat	Total testis cholesterol mg./100 gm. of rat	Ester cholesterol mg./gm. testis (wet wt.)	Ester cholesterol of testis mg./100 gm. of rat
9	Saline 6 days	65 ± 2.0	907 ± 24.0	20.9 ± 1.01	1.82 ± 0.06	0.07 ± 0.02	0.07 ± 0.02
5	LH 6 days	63 ± 1.3	974 ± 47.6	**39.7 ± 1.99**	**2.15 ± 0.06**	**0.37 ± 0.06**	**0.34 ± 0.05**
5	Saline 6 days Prolactin 4 days	**85 ± 3.1**	**1086 ± 39.6**	**32.2 ± 1.31**	1.98 ± 0.11	0.09 ± 0.02	0.10 ± 0.02
6	LH 6 days Prolactin 4 days	**84 ± 1.7**	988 ± 34.0	**29.3 ± 2.14**	1.83 ± 0.08	0.09 ± 0.02	0.08 ± 0.02
9	LH 6 days Saline 4 days	**81 ± 3.5**	**1033 ± 21.0**	25.1 ± 1.52	1.96 ± 0.04	0.12 ± 0.02	0.13 ± 0.02

increased both the testis cholesterol concentration and its absolute amount. Saline-prolactin failed to change the cholesterol ester, although it is possible that the prolactin preparation was contaminated with a small amount of LH in view of the rise in seminal vesicle weight. As in the preceding experiment, LH treatment followed by saline resulted in a marked drop in cholesterol ester from the level attained after 6 days of LH treatment. A similar fall occurred after prolactin treatment, and, although there is a trend toward further fall in the latter group, neither figure is significantly different from the control value.

DISCUSSION

On the basis of analytical and histochemical studies of adult, hypophysectomized and cryptorchid rats Perlman (1950) concluded that "there appears to be no readily discernible interrelationship between testis cholesterol level and/or the ester/free cholesterol ratio and interstitial cell function." In the present study, in which weanling, intact rats and immature rats implanted with stilbestrol pellets were used, a readily reproducible increase in testis cholesterol ester was demonstrated with a

TABLE V. A SUMMARY OF THE EFFECTS OF SEVERAL GONADOTROPHINS ON THE CHOLESTEROL ESTER CONTENT OF THE TESTIS OF IMMATURE, INTACT RATS

No significant change in testis cholesterol ester	Significant increase in testis cholesterol ester
Chorionic Gonadotrophin (Squibb)	LH FW234 (Armour)
Chorionic Gonadotrphin (Ayerst)	LH (McShan-Meyer)
PMS (Schering)	LH + FSH (McS-M)
Prolactin (Schering)	Gonadophysin (Searle)
FSH (McShan-Meyer)	"FSH Synergist" (Schering)

number of different gonadotrophin preparations of pituitary origin, but not with chorionic gonadotrophin (see Table 5). This increase in cholesterol ester was, in all instances, associated with augmented androgen output as indicated by seminal vesicle weight increases. Moreover, the significance of the analytical evidence of an increase in testis cholesterol ester was enhanced by the finding that treatment with purified pituitary LH preparations caused a marked increase in the sudanophilic lipid content of the interstitial cells of the testis. These cells, according to current theory, may be regarded as the immediate target cells of pituitary LH in the male. These changes observed in the testis are in the same direction as were those observed in the corpus luteum by Everett, (1947), who used a McShan-Meyer LH preparation in his studies. The fact that the same hormone produced the same type of chemical morphological change in the tissues of male and female animals suggests the possibility that the basic mechanism of action of the hormone may be similar in the two sexes. Since it is the esterified fraction of the cholesterol which varies reproducibly with LH administration, and since it is esterified cholesterol which fluctuates widely in the adrenal cortex in association with alterations in rate of hormone production, this fraction may well contain the steroid hormone precursor. If this is the case, trophic regulation of steroid hormone production may involve rate regulation of esterified cholesterol concentration either by promoting esterification of pre-existing cholesterol in the gland or by favoring the abstraction of preformed cholesterol ester from the blood plasma which traverses the gland. Indeed, Conn and Vogel (1949) in their studies of changes in esterified cholesterol in the serum of patients treated with ACTH, stress the potentially limiting character of the rate of esterification of cholesterol in determining the secretory performance of a steroid-producing gland.

On the other hand, the testis may increase its output of androgen strikingly without any detectable increase in its content of esterified cholesterol. This is the case in animals treated with chorionic gonadotrophin. In fact, in rats pre-treated with gonadophysin, chorionic gonadotrophin causes a decrease in esterified cholesterol. On the basis of these striking differences in effect on the chemical morphology of the testis it is reasonable to suggest that LH and CG may well stimulate androgen production by influencing entirely different reactions in the chain from accumulation of hormone precursor to secreted hormone.

SUMMARY

A purified pituitary FSH preparation was without effect on the cholesterol ester content of the testes of intact, weanling rats. Injection with several purified pituitary LH preparations markedly increased the

cholesterol ester content of the testes of comparable animals. Qualitatively similar results were obtained with stilbestrol pellet-implanted rats. Although it caused a marked enhancement of androgen output as indicated by seminal vesicle weight increases, chorionic gonadotrophin did not cause an increase in testis cholesterol ester. Attempts to demonstrate effects of chorionic gonadotrophin and of prolactin on the ester cholesterol content of the testes of LH pre-treated rats are described.

A summary of the effects of various gonadotrophins on testis cholesterol ester is given. Only those materials which contain LH of pituitary origin caused an increase in testis cholesterol ester.

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PROTEIN-BOUND IODINE IN VARIOUS TISSUES AFTER INJECTION OF ELEMENTAL IODINE[1]

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IN 1947, Dvoskin reported that the subcutaneous injection of elemental iodine returned toward normal the depressed growth rate of young thyroidectomized rats and lowered the cell height of the thyroid epithelium of thiouracil-treated animals. Both of these effects were interpreted as being due to the formation of thyroxine, but no demonstration of organic iodine formation was made. Nelson and Wheeler (1948) found that the disturbances of the estrous cycle produced in rats by thyroidectomy could be reversed by elemental iodine injections.

Barker and Lipner (1948) showed an elevation of metabolic rate in thyroidectomized or thiouracil-treated rats injected with 16 mg. I_2 per kilogram body weight per day and in thyroidectomized injected with 4; normal animals showed no effects at either dose level. The protein-bound iodine (PBI) of the blood plasma increased remarkably in all groups of animals to a greater extent than would be expected from the metabolic changes. The present work was undertaken to inquire further into the PBI changes in various tissues.

METHODS

Male albino rats of the Sprague-Dawley strain weighing from 250 to 350 gm. were used, all animals being fed the same commercial diet (Rockland). Thyroidectomies were performed under ether anesthesia, with at least one month elapsing for recovery from the operation and for full development of hypometabolism. Thiouracil-treated rats received the drug[2] admixed with the food, 2 gm. being thoroughly distributed throughout one kilo of ground diet. This resulted in the ingestion of about 100 to 150 mg. of thiouracil per kilo of body weight per day.

Elemental iodine was dissolved in propylene glycol, thus avoiding the need for adding NaI, and injected subcutaneously once per day for 28–30 days, at either 4 or 16 mg. per

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[2] Thiouracil used in these studies was generously furnished by Dr. Stanton M. Hardy of the Lederle Laboratories, Pearl River, N. Y.

kilogram of body weight. Control animals of each group, normal,[3] thiouracil-treated or thyroidectomized, were injected with propylene glycol alone. Care was taken with all animals to rotate the site to avoid undue irritation in any one location.

PBI determinations were made according to Barker (1948), with the modification of using arsenite in the trap of the still instead of sulfite (Barker and Lipner, 1948). The tissues were homogenized, proteins precipitated by the Somogyi zinc hydroxide procedure, washed free of any contaminating inorganic iodide and analyzed. When the animals had received I_2, appropriate aliquots of the thoroughly suspended homogenates of such tissues as site of injection, kidney and liver were added to dog plasma of known PBI content as a convenient "carrier" before precipitation of proteins. Butanol-soluble iodide (BuI) was determined on plasma by a modification of Taurog and Chaikoff's procedure (1948), in which 2 cc. of plasma were acidified to pH 3.5 with NH_2SO_4 and extracted with, successively, 6, 4 and 4 cc. of n-butanol. The 14 cc. of butanol were washed three times with 7 cc. of Na_2CO_3-NaOH mixture to remove diiodotyrosine and inorganic iodide. Following complete removal of the butyl alcohol in a boiling water bath, protein carrier of known I content was added and the BuI determined. Recovery of added thyroxine was 85–95% complete and added NaI and diiodotyrosine were better than 99% eliminated. For purposes of discussion, therefore, BuI can be considered as thyroxine I, although discrepancies are apparent at low PBI levels.

TABLE 1. PROTEIN-BOUND IODINE IN VARIOUS TISSUES OF THE RAT AS ALTERED BY SUBCUTANEOUS INJECTION OF I_2

	Normal*			Thiouracil*			Thyroidect.*		
	PG	4	16	PG	4	16	PG	4	16
Kidney μgm./100 gm.	32.6	392.1	2893	21.7	675.0	3149	29.6	720.6	3507
Liver μgm./100 gm.	25.9	44.9	166.3	27.1	57.5	166.4	27.2	72.0	132.3
Diaphr. μgm./100 gm.	18.0	32.8	65.0	13.8	37.6	77.0	17.9	45.0	64.1
Heart μgm./100 gm.	21.5	45.4	72.3	17.3	38.5	97.9	20.3	45.8	78.1
Skel. m. μgm./100 gm.	9.5	10.7	28.1	7.9	13.4	34.8	8.8	18.6	31.9
Site inj. μgm./100 gm.	9.0	121,581	193,789	9.4	55,418	211,025	11.7	56,847	199,164
Plasma μgm./100 cc.	5.4	78.3	262.1	1.6	64.3	271.3	1.6	102.0	289.0
Thyroid μgm./gland	21.9	25.1	24.1	2.1	3.4	4.4	—	—	—

* The values shown in this table are averages of 4 to 6 animals in each of the 9 groups. The injections indicated in the next line are, respectively, propylene glycol. 4 mg. I_2 and 16 mg. I_2 per kilo per day for 28–30 days.

RESULTS AND DISCUSSION

The tissue PBI values are summarized in Table 1. It can be seen, as pointed out previously by Barker and Lipner (1948), that the level at the site of injection was much higher than that of any other tissue studied. One may infer from this finding that the elemental iodine reacted directly with protein to form a reservoir of PBI which was absorbed into the blood plasma, perhaps via the lymph, and then reached the rest of the body. This conclusion is supported by the plasma PBI's which were higher than other tissues except kidney. It seems unlikely that formation of PBI would take place in kidney following subcutaneous injection of I_2. A probable explanation is that the plasma PBI or metabolic products of it accumulate in the kidney during excretion.

[3] The term "normal" is used to denote litter-mate animals not treated with thiouracil or thyroidectomized.

The increasing levels of PBI with 4 and with 16 mg. I_2 in most of the tissues appear quite comparable for each group of animals, whether normal, thiouracil-treated or thyroidectomized. Thus, the greater metabolic response obtained earlier from thyroidectomized rats by Barker and Lipner would be expected to have been due to their greater sensitivity rather than to any quantitative differences in formation of PBI. However, determinations of plasma BuI levels indicate that the explanation may not be so simple. Table 2 contains the unexpected finding that the thyroidectomized animals had higher circulating thyroxine values at both I_2 doses than either normal or thiouracil-treated. Moreover, since the starting BuI levels for the hypothyroid rats were low, the iodine-

TABLE 2. FRACTIONATION OF PLASMA I (μGM. %)

	Animals	Total	PBI	BuI
PG*	Normal	116.0	8.3	6.2
	Thiouracil	67.2	2.8	3.2
	Thyroidectomized	135.6	2.2	2.3
4 mg. I_2†	Normal	173.0	67.8	6.7
	Thiouracil	254.7	68.5	6.1
	Thyroidectomized	174.0	116.7	8.7
16 mg. I_2†	Normal	376	243.5	9.0
	Thiouracil	492	264.7	9.2
	Thyroidectomized	476	277.0	13.0

* Averages are shown of 4 pooled samples from 8 rats in each of the 3 groups given PG. All determinations were performed on each of the 12 composite mixtures.
† Averages are shown of 4 individual samples in each of these groups.

induced increases in the case of the thyroidectomized become still more striking. At the present time, it is impossible to say whether these differences could in themselves account for the metabolic changes. Perhaps the conservative conclusion for the time being would recognize both higher circulating thyroxine level and metabolic sensitivity to the hormone as participating in the effects seen.

Table 3 presents the changes in thyroid weights and histological appear-

TABLE 3. CHANGES IN RAT THYROID GLANDS FOLLOWING I_2 INJECTIONS FOR 28–30 DAYS

	Animals	Thyroid weights (mg./kg.)	Thyroid histology
PG	Normals (11)	85.3	Reference.
	Thiouracil (9)	399.6	High columnar epithelium, colloid exhaustion, some hyperplasia.
4 mg. I_2	Normals (8)	67.1	6 no differences from controls, 2 flattened epithelium.
	Thiouracil (8)	223.6	No differences from thiouracil controls.
16 mg. I_2	Normals (12)	70.3	11 flattened epithelium, 1 same variations as in controls.
	Thiouracil (8)	168.9	2 no differences from the thiouracil controls, 3 considerable decrease in cell height and colloid storage, 2 marked return toward normal.

ance produced by the elemental iodine. The expected differences were seen between normals and thiouracil-treated on propylene glycol, including larger gland, lower PBI content (Table 1), high columnar epithelium and colloid exhaustion. The lower dose of 4 mg. I_2 per kg. per day for 3 to 4 weeks produced a 21% decrease in the normal animals' gland weight and 34% in the thiouracil-hypothyroid. There was some increase in the iodine content, although the histology was hardly altered. The differences may have been due to a decrease in vascularity, although no attempt was made to quantitate this. With the 16 mg. quantity of I_2, marked histological changes indicating a return toward normal were obtained in both groups of animals, together with a continued decrease in gland size and increased PBI content in the thiouracil-treated.

SUMMARY

Tissue protein-bound iodine levels have been measured after 4 weeks' injections of elemental iodine at two dose levels, 4 and 16 mg. I_2 per kilogram body weight per day, into normal, thiouracil-treated or thyroidectomized rats.

The findings are compatible with the concept that the iodine reacts with protein directly at the site of injection to form a type of PBI low in thyroxine which is absorbed into the blood plasma and distributed to the various other tissues. The kidney level of PBI becomes higher than any other away from the injection site, perhaps because of accumulation of material being excreted.

The previously encountered greater metabolic response of the thyroidectomized rats over the others may be explicable partly on the basis of a greater sensitivity, but the higher plasma thyroxine level found in these animals must also be taken into consideration.

Changes in thyroid gland size, colloid storage, iodine content and histology were marked only with the larger iodine dosage, although some effects were produced by the smaller.

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THE ACUTE EFFECT OF ADRENAL CORTICAL EXTRACT, ADRENOCORTICOTROPIC HORMONE, AND DESOXYCORTICOSTERONE ACETATE ON THE THIOCYANATE, BROMIDE, AND MANNITOL SPACES; PLASMA VOLUME AND TOTAL BODY WATER IN NORMAL DOGS

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INTRODUCTION

IT HAS long been accepted that there are striking shifts of fluid as well as electrolytes between the intracellular and extracellular fluid compartments under the influence of the adrenal cortex. The plasma and interstitial spaces—subdivisions of the extracellular compartment—also undergo changes.

In Addison's disease there is an associated loss of sodium and chloride as well as water through the kidney and later the gastro-intestinal tract (Harrop et al., 1933). Retention of potassium by the kidney as well as an overall increase in the intracellular and extracellular potassium takes place (Harrison and Darrow, 1938; Buell and Turner, 1941; Hegnauer and Robinson, 1936). There is a fluid depletion of the plasma and interstitial spaces, subdivisions of the extracellular compartment, with a reduction in their volume and at the same time cellular overhydration with an increase in the size of the intracellular (Harrop *et al.*, 1933; Clarke and Cleghorn, 1942). Certain corticosteroids, especially desoxycorticosterone acetate, may cause retention of sodium, chloride and water by the kidney (Anderson, 1943; Clinton *et al.*, 1942). Recent work by Zaffaroni *et al.*, (1950) using paper chromtography has confirmed the findings of Reichstein by demonstrating the presence of this hormone in commercial adrenal cortical extract as well as in the perfusates of beef adrenals (Pincus *et al.*, 1951). There is a resulting increase in the size of the extracellular compartment and eventually even the intracellular compartment. Edema and hypertension may result (Freidman *et al.*, 1948). These changes have been measured many times, but the experiments have extended over several

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days (Gaudino and Levitt, 1949; Mach and Odier, 1946). Under these circumstances the known renal actions of the adrenal cortical steroids as well as their possible action on the gastro-intestinal absorption of fluids and electrolytes become the major factors in causing body fluid abnormalities (Boss *et al.*, 1950).

It is possible that the adrenal cortical steroids have a direct effect on the capillary membrane, the cell membrane, or both. Studies have attempted to show that capillary permeability is increased by adrenal cortical insufficiency, but the results have not been without question (Cope *et al.*, 1942; Freed and Lindner, 1941; Menkin, 1951). Very little evidence is at hand concerning the effect of the adrenal cortical steroids on the cell membrane.

Water intoxication experiments have shown an extrarenal action of adrenal steroids, the exact mechanism of which is not known. It is thought, however, that cellular overhydration is partially prevented by the adrenal cortical steroids (Birnie *et al.*, 1947). Cortical extract will sometimes revive adrenalectomized animals in collapse without additional fluid or electrolytes (Hartman and Brownell, 1930). This indirect evidence indicates that a readjustment of the fluid and electrolytes takes place within the body after administration of adrenal cortical extract.

The studies outlined in this paper were designed to measure any acute change in the size of the fluid compartments of the body produced by the intravenous injection of several adrenal steroids and adrenocorticotropic hormone. By limiting the time for reaction, the renal and gastro-intestinal effects of the hormones tested did not have time to alter the fluid and electrolyte balance to a measurable degree. Thus the changes produced—subject to the technical limitations outlined below—can be attributed to a direct effect of the cortical hormones on peripheral tissues.

METHODS

In the following discussion the term, reference substance, indicates one of the materials used to measure one of the fluid spaces, namely SCN^-, Br^-, Mannitol, T-1824, and deuterium. Zero time is the time of injection of this reference substance. The term, hormones tested, refers to whole adrenal cortical extract, desoxycorticosterone acetate and adrenocorticotropic hormone.

Large dogs (18–30 kg.) were stabilized as to weight, plasma volume, and thiocyanate space. They were kept on a standard diet and observed during the fall, winter, and spring months only, at which time their environment was essentially constant. The period of observation varied from 3 months to 18 months with no determinations being done during the summer. The usual practice was to perform one control and one experimental determination weekly for three weeks and then rest the animal one week. The cycle was then repeated. During the period of standardization the dogs were trained to undergo the procedure without excitement.

In order to standardize as nearly as possible the state of hydration of the animals for

all experiments the following steps were taken. Zero time was always the same time of day, 1:00 P.M. The dogs were fasted for 24 hours prior to zero time, but allowed water ad libitum until 8:00 A.M. of the morning of the experiment. Both food and water were then withheld until the end of the experiment, usually 4:00 P.M.

On most occasions simultaneous determinations of thiocyanate space, plasma volume and either mannitol or bromide space were carried out. Numerous control experiments were performed to show that none of the reference materials interfered with the simultaneous determination of any other reference material.

Total body water was determined by the dilution of deuterium oxide. Measurement of the deuterium oxide concentration was done by the falling drop method (Keston *et al.*, 1937).

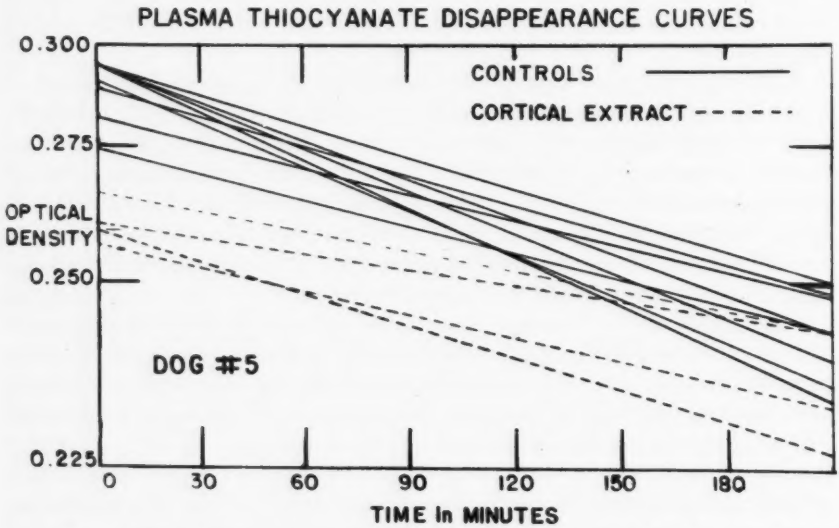

FIG. 1. Plasma Thiocyanate Disappearance Curves. Each straight line curve is constructed from optical density measurements made every 30 minutes for 3 hours plotted against time.

The thiocyanate space was determined by the method of Crandall and Anderson (1934), adapted to spectrophotometric analysis. The brown color of the ferric thiocyanate was measured at a wave length of 480 Angstrom units. The data presented are based on samples drawn 2 hours after zero time. The slope of the disappearance curve of thiocyanate varies in different dogs. There is also a variation from day to day in the same dog (See Fig. 1). The slope variation is, however, small, and it is a random variation bearing no relation to the urinary output of SCN^-, the state of hydration of the dog or other observed factors. Examination of Figure 1 reveals that extrapolation to zero time would have reduced the standard deviation of the control values. This would have necessitated the collection of many additional data and is only reported for this dog.

The simultaneous determination of the thiocyanate space and the plasma volume with the dye, T-1824, was done according to the method of Gregersen and Stewart (1939). The plasma volume was determined from the plasma concentration at zero time

by extrapolation. The total blood volume was not calculated, but was normal. The hematocrits varied from 40 to 50 per cent in the various animals. Due to a wide variation between the A.M. and P.M. hematocrits during the control determinations, no definite conclusion as to the effect of the hormones tested on the hematocrit could be made.

The mannitol space was determined spectrophotometrically according to the method of Corcoran and Page (1947). Straight line plasma disappearance curves were obtained by plotting plasma concentration versus time on semilogarithmic graph paper. The plasma mannitol concentration at zero time, found by extrapolation, was used in the calculations. The slopes of the disappearance curves of mannitol varied slightly from day to day in the same dog and from dog to dog in what appeared to be a random manner. The slopes of the disappearance curves after cortical extract, desoxycorticosterone acetate and adrenocorticotropic hormone injection also varied from day to day, but were within the range of variation of the control slopes. It seems therefore that for the dosage used, the three hormone preparations tested had no effect on the rate of urinary excretion of mannitol.

Sodium and potassium determinations were done by flame photometry.

Plasma bromide determinations were done by spectrophotometric determination of the iodine color developed according to the method of bromide analysis of Friedman (1942). The bromide space was determined from the plasma bromide level 3 hours after sodium bromide injection.

One hundred and sixty-two experiments were performed, all involving determination of several fluid spaces. The last one hundred and one consecutive experiments are reported here.

Adrenal cortical extract was given intravenously 4 hours before zero time (the time of injection of the reference substance, SCN^-, etc.). On two occasions the extract was given 1 hour before zero time with similar results.

Crystalline desoxycorticosterone acetate was given intravenously 2 and 4 hours before zero time. The time interval produced no significant difference, and so the results have not been separated in the tables. The steroid was dissolved in propylene glycol or given as an emulsion in water. Since desoxycorticosterone acetate in propylene glycol solution is immediately precipitated upon its injection into the blood, there is no advantage in using this method of administration. Propylene glycol alone was given during a group of the control experiments without effect. No embolic phenomena were noted.

Adrenocorticotropic hormone (Armour) with a potency of 180 per cent of Armour's standard L-A-1-A preparation was dissolved in distilled water immediately before use and injected intravenously 4 hours before zero time. Injection intravenously 1 and 8 hours before zero time as well as intramuscularly 4 and 8 hours before zero time, produced no deviation from the control values, as was the case with most of the 4 hour experiments. There was a reduction in eosinophiles of greater than 80 per cent 4 hours after injection of adrenocorticotropic hormone in all cases. Counts were done according to the method of Roche *et al.* (1950).

RESULTS

Numerous control determinations (column 2 of the tables) were averaged to give the control means (column 3) for each dog. These are reported as cc./kg. so that results from different animals will be comparable. Standard deviations (column 4) for each set of control values are reported. The experimental results are reported as deviations from the control

mean (columns 6, 8, 10). A positive value means an increase in the size of the fluid space following hormone injection, a negative value the reverse. Evaluation of the significance of the experimental results by statistical analysis is not possible because of the type of data available. Despite the large total number of determinations each dog must be considered separately. However a change in the volume of a given space is probably significant if it exceeds the standard deviation of the control values for that animal. For example, in Table I, column 6, the first experiment on dog #1 gave an increase in the thiocyanate space of 47 cc./kg. which exceeds the standard deviation of the control values for that animal, ±12 cc./kg., given in column 4.

TABLE I. DEVIATION OF THE THIOCYANATE SPACE (AVAILABLE FLUID) FROM CONTROL MEAN

1	2	3	4	5	6	7	8	9	10
Dog # and sex	Number of control experiments	Mean control value SCN space cc./kg.	Std. dev. control SCN space cc./kg.	I.V. cortical extract		I.V. DOCA		I.V. ACTH	
				Quan. inj. cc./kg.	Dev. SCN space cc./kg.	Quan. inj. mgm./kg.	Dev. SCN space cc./kg.	Quan. inj. mgm./kg.	Dev. SCN space cc./kg.
1	16	377	±12	0.8	+47	0.3	+20	1.3	+14
(A)				0.8	+28			1.3	+13
				0.8	+42				
(M)				1.6	+23				
				0.8	+25				
1	4	395	±6		—	0.8	+22	1.3	−11
(B)						1.6	+1		
2	9	423	±9	1.0			—	1.7	+9
(M)				1.0	+32				
				0.8	+30				
3	18	390	±14	0.7	+21	0.5	+5	0.8	−22
				0.7		0.5	−3	1.7	−6
				1.7	+66	0.7	+8	1.7	−3
(M)						1.7	+24	0.8	−1
						1.7	−8	1.6	+27
						8.5	+1		
4	7	353	±16	0.7	+55	1.3	−4	1.1	−10
(M)				0.7		2.2	+1		
5	15	388	±5	0.7	+36	0.7	−5	1.2	−1
				0.7	+35			1.2	−10
				0.7	+17				
(M)				1.4	+6				
				0.7	+8				
6	6	341	±6	0.9	+19		—	1.7	+6
(F)				0.7	+14			3.9	−4
				2.0	+25				

The number of control experiments performed on each dog is given in column 2. The mean control thiocyanate space for each dog is given in column 3, and the standard deviation of the mean in column 4. The dosage schedule and the deviation of the thiocyanate space from the control mean after hormone injection are shown in columns 5 to 10.

Thiocyanate Space (see Table I): The control values for each dog were closely grouped. The standard deviations of the control values for the various dogs represent from 1.3 per cent to 4.5 per cent of the control means. It can be seen from column 6 that all deviations of the thiocyanate space following adrenal cortical extract administration are positive and all exceed the standard deviation of the control values for that dog. The

deviations after desoxycorticosterone acetate administration are both positive and negative (column 8). There are three positive deviations which exceed the standard deviation of the applicable control values. The results of adrenocorticotropic hormone stimulation show a much wider variation (column 10). There are three positive and three negative deviations which exceed the standard deviation of the applicable control values. There are, however, only two large deviations, one positive and one negative.

TABLE II. DEVIATION OF THE T-1824 SPACE (PLASMA VOLUME) FROM THE CONTROL MEAN

1	2	3	4	5	6	7	8	9	10
Dog # and sex	Number of control experiments	Mean control value T-1824 space cc./kg.	Std. dev. control values T-1824 space cc./kg.	I.V. cortical extract		I.V. DOCA		I.V. ACTH	
				Quan. inj. cc./kg.	Dev. T-1824 space cc./kg.	Quan. inj. mgm./kg.	Dev. T-1824 space cc./kg.	Quan. inj. mgm./kg.	Dev. T-1824 space cc./kg.
1 (M)	7	72	±2	0.7 0.8 0.8	+2 +1 +3				
2 (M)	3	71	±2	1.0	0				
3 (M)	9	67	±1.3	1.7	+10	0.5 0.7 1.7 8.5	−1 −1 +6 −3	1.7 1.7	−0.2 −0.7
5 (M)	7	71	±2	0.7 0.7 0.7 1.4	+2 −0.7 +2.6 −0.5				

The number of control mannitol space determinations performed on each dog is given in column 2. The average of the control values for each dog is given in column 3, and the standard deviation of these control values in column 4. The dosage schedule and the deviation of each mannitol space determination from the control mean after hormone injection are shown in columns 5 to 10. All values are given in cc. or mg. per kilogram of body weight.

Plasma Volume (see Table II): The control values for each dog were very closely grouped. The standard deviations of the control values for the various dogs represent from 1.9 per cent to 2.8 per cent of their respective control means. Despite these small standard deviations there were only five experimental deviations which exceeded the standard deviation of the respective control values. However, only two deviations appear significant. Following the largest dose of adrenal cortical extract a significant positive deviation was noted (dog #3, column 6). After one of the larger quantities of desoxycorticosterone acetate a significant positive deviation was noted. An even larger quantity of desoxycorticosterone acetate, however, resulted in a small negative deviation (dog #3, column 8).

Mannitol Space (see Table III): The control values for dog #3 show a standard deviation of ±20 cc./kg. This represents 7.3 per cent of the mean control value. Despite this moderate spread of the control values, the determinations after adrenal cortical extract exceeded the standard deviation in both cases (column 6). Dog #6 showed a standard deviation of only 2.7 per cent of the control mean. Adrenal cortical extract produced

TABLE III. DEVIATION OF THE MANNITOL SPACE (AVAILABLE FLUID) FROM CONTROL MEAN

1	2	3	4	5	6	7	8	9	10
Dog # and sex	Number of control experiments	Mean control value mannitol space cc./kg.	Std. dev. control mannitol space cc./kg.	I.V. cortical extract		I.V. DOCA		I.V. ACTH	
				Quan. inj. cc./kg.	Dev. mannitol space cc./kg.	Quan. inj. mgm./kg.	Dev. mannitol space cc./kg.	Quan. inj. mgm./kg.	Dev. mannitol space cc./kg.
3 (M)	6	273	±20	0.7 1.7	+26 +72	1.7 8.5	+4 +4	1.7	+4
6 (F)	4	258	±7	0.7 2.0	+20 +20	—	—	1.7 3.9	+51 +6

The number of control experiments performed on each dog is given in column 2. The mean control mannitol space for each dog is given in column 3, and the standard deviation of the mean in column 4. The dosage schedule and the deviation of the mannitol space from the control mean after hormone injection are shown in columns 5 to 10.

a positive deviation which exceeded the standard deviation in all cases (column 6). These changes in volume were of approximately the same magnitude as those demonstrated by the simultaneously determined thiocyanate space. Desoxycorticosterone acetate produced a very small deviation much less than the standard deviation (column 8). Adrenocorticotropic hormone produced no significant deviation on two occasions, but a third trial resulted in a large positive deviation (column 10).

The *bromide space* determinations are not reported because of the small number of experiments. The data does, however, seem to indicate an increase in the bromide space after adrenal cortical extract, but no change after desoxycorticosterone acetate and adrenocorticotropic hormone.

Total body water: In the normal animal the total body water remains a relatively constant proportion of the body mass and can be closely estimated from the weight of the animal. By desiccation experiments the total body water in the dog has been shown to be approximately 65 per cent of the fat free body weight (Fulton, 1947). During the observation period the weight of the animals was kept as nearly constant as possible. The change in weight during the course of an experiment (4–8 hours) was negligible. Determination of the total body water by deuterium oxide before and 4 hours after injection of adrenal cortical extract revealed no measurable change. Three additional total body water determinations were carried out on the same animal over a period of 1 month. The insignificant changes measured by this method were anticipated quite well by similar changes in the animal's weight.

Intracellular fluid compartment. The changes in this compartment have not been included in the tables. Since the total body water was shown to remain essentially constant, this space can be determined by subtraction of the extracellular fluid volume from the total body water (65 per cent of the animal's weight). The changes in the intracellular fluid volume are numerically the same as the changes in the extracellular fluid volume, but with an opposite sign.

The sodium thiocyanate output in the urine during the first 3 hours after injection (zero time) was measured in two dogs. In dog #3, the control values were 3.42, 3.75, 4.49, 21.2, 26.2, and 45.1 mgm. After adrenal cortical extract, the values were 7.82 and 21.0 mgm.; after adrenocorticotropic hormone 24.2 mgm.; after desoxycorticosterone acetate 34.7 mgm. In dog #4, control values were 1.8 and 31.0 mgm. After adrenal cortical extract 14.5 mgm. were excreted. These values represent excretion of from 0.1 to 1.5 mgm. per kg. of body weight of sodium thiocyanate during the period allowed for equilibrium of this reference material. Note that all experimental values fall within the range of the control values. It is thus evident that the rate of urinary output of thiocyanate was variable, but not effected by the hormones tested.

The plasma electrolyte concentrations were measured before injection of the hormone to be tested (9:00 A.M.), before injection of the reference material (1:00 P.M.), and at 4:00 P.M. on the day of the experiment. The determinations were repeated the following day at 8:00 A.M.

There were no significant changes in either the sodium or potassium concentrations on the day of the experiments. This was true of both the control experiments and those following injection of one of the hormones to be tested. On the morning following the injection of adrenal cortical extract and desoxycorticosterone acetate there was a drop in the serum potassium concentration. The initial concentrations were 4.5, 4.5, 4.5, and 4.6 m.eq./l. Twenty-four hours after injection of adrenal cortical extract the serum potassium was found to be 2.5 m.eq./l on three occasions. Following desoxycorticosterone acetate injection it was 2.9 m.eq./l.

Adrenocorticotropic hormone produced no change in the plasma electrolytes during the 24 hours of observation.

DISCUSSION

The data presented in the tables show a consistent increase in the thiocyanate, mannitol and bromide spaces after adrenal cortical extract injection. Since the total body fluid volume was essentially unchanged during the course of the experiments, there seems to have been a diffusion of fluid from the intracellular to the extracellular fluid compartment. The thiocyanate, mannitol and bromide spaces are calculated from the concentration of the reference material in the plasma after equilibrium has been reached. The size of the thiocyanate, mannitol or bromide space is inversely proportional to the concentration of the reference material in the plasma. Other factors which might cause a decrease in the plasma concentration of the reference material must be considered before one can assume that the apparent increase in the size of the thiocyanate, mannitol and bromide spaces is due to a diffusion of fluid from the intracellular to the extracellular

compartment. There are several mechanisms by which the plasma concentration of the reference material might be decreased.

1). Following injection of adrenal cortical extract a larger quantity of the reference material may enter the red blood cells than is the case during control experiments. Previous reports have shown that mannitol does not enter the red blood cells at all (Smith *et al.*, 1940; Newman *ei al.*, 1944). A small amount of thiocyanate enters the red blood cells. In order to evaluate the effect of adrenal cortical extract on the rate of diffusion of thiocyanate into red blood cells the following experiment was performed: Tubes of whole dog blood containing sodium thiocyanate and adrenal cortical extract were incubated for three hours at 37°C. The thiocyanate concentration was similar to that obtained in the dogs; several concentrations of adrenal cortical extract were used. After incubation the concentration of thiocyanate in the plasma was compared to that in control tubes containing whole blood and thiocyanate, incubated in a similar manner. The thiocyanate concentration in the plasma was determined spectrophotometrically as previously described. Although some thiocyanate slowly diffused into the red blood cells, the rate of diffusion was unaltered by the adrenal cortical extract. Since the rate of diffusion is unchanged, this factor has no effect on the measurement of changes in volume of the thiocyanate space.

2). Under the proper conditions certain materials become protein bound. This binding action of the protein might be increased by adrenal cortical extract. An increased quantity of reference material would then be lost when the protein was removed by precipitation in the course of determination. Neither thiocyanate nor mannitol have been reported to combine chemically or physically with protein. The in vitro experiment described in the above paragraph showed that the adrenal cortical extract has no effect on the quantity of thiocyanate which is protein bound. If the quantity of protein bound thiocyanate had been increased by adrenal cortical extract, the calculated concentration of thiocyanate in the tube containing adrenal cortical extract would have been less than in the control tube. Such was not the case as the thiocyanate concentrations remained identical.

3). The capillary wall is a semipermeable membrane. The diffusion gradient across this membrane (permeability) may be altered by adrenal cortical extract. Because of the inaccessibility of the interstitial fluid, direct evaluation of this factor was not attempted. However, it seems unlikely that three substances, so widely different in nature and molecular size, would be effected in the same manner.

4). If the reference material is metabolized by the body, the rate of metabolic destruction may be altered by adrenal cortical extract. Thiocyanate and bromide pass through the body unchanged. Some authors have reported that mannitol is inert, while others have found that a small

portion of the quantity injected is metabolized (Carr *et al.*, 1933; Todd *et al.*, 1939; Smith *et al.*, 1940; Elkinton, 1947; Corcoran *et al.*, 1947; Houck, 1951). However, unless the *rate* of metabolism was altered, the loss of mannitol in this manner would not effect the measurement of a change in fluid volume since an equal quantity of mannitol would be lost during control experiments.

5). All the reference materials used in this study are almost completely excreted through the kidneys. A small portion of the mannitol is probably metabolized. The urinary excretion of bromide is slow, extending over several weeks, while thiocyanate is excreted over a period of days. Mannitol is more rapidly excreted over a period of hours. An immediate increase in the rate of excretion of the reference material following adrenal cortical extract injection would lower the plasma concentration of the reference material. Measurements of the urinary excretion of thiocyanate were made. The control experiments showed a slight variation in the quantity of thiocyanate excreted during the four hour period following injection. There was no significant change in the quantity of thiocyanate excreted after simultaneous injection of adrenal cortical extract.

6). The cell wall is a semipermeable membrane which separates two fluid compartments. Diffusion of a reference material across this boundary would result in a decrease in the plasma concentration of the reference material. Thiocyanate is known to diffuse slowly into red blood cells and probably into other cells. Mannitol is a much larger molecule and probably does not enter the cells. The collateral experiment described above shows that adrenal cortical extract has no effect on the diffusion of thiocyanate into red blood cells. A second collateral experiment was performed on rats in order to measure the effect of adrenal cortical extract on the diffusion of thiocyanate into living cells. One group of rats was given a quantity of sodium thiocyanate comparable on a weight basis to that given to the dogs. The second group of rats was given a similar quantity of sodium thiocyanate plus adrenal cortical extract. There was no significant difference in the thiocyanate concentration of the muscle of the two groups 4 hours after injection. Therefore adrenal cortical extract apparently has no effect on the rate of diffusion of thiocyanate into the cell.

7). Water exchange in dense connective tissue such as tendons is unusually slow. Equilibrium with plasma is frequently not reached for several days. If the rate of diffusion into these remote parts of the interstitial space were increased by adrenal cortical extract, then the plasma concentration of the reference material would fall. Tissues from the rats used in the collateral experiment already described were dried and weighed. The water content of striated muscle, liver, heart, spleen, adipose tissue and tendon was determined in the control and experimental groups. The only change

noted after adrenal cortical extract injection was an increase in the water content of the tendons. It increased from 22.4 to 28.0% of the net weight. If this increase in water content of the dense connective tissue was accompanied by an increase in the rate of diffusion of the reference material into the dense connective tissue, then this factor would lower the plasma concentration of the reference materials.

8). The primary assumption which has guided this work is that the fall in the plasma concentration of the reference material is due to an increase in the size of the extracellular fluid compartment. If the possibilities outlined above are excluded, then this assumption is justified. Although the various possibilities outlined above have not been completely excluded, the divergent nature of the reference materials make it quite unlikely that they would all be effected in a similar manner. In the following discussion it is assumed that changes in the plasma concentration of the reference material indicate changes in the size of the extracellular fluid compartment.

It is well known that thiocyanate, mannitol and bromide do not measure the exact extracellular fluid compartment. However, when a change in the size of the extracellular fluid compartment is produced, such as by an infusion of 0.9 percent sodium chloride solution, the calculated thiocyanate, bromide or mannitol space will increase by an amount almost identical with the volume of fluid injected (Winkler *et al.*, 1943). It is with such a change in the extracellular fluid volume that this paper is concerned.

The plasma space is a subdivision of the extracellular fluid compartment. One might, therefore, expect an increase in the size of the plasma space concurrent with the increase in the size of the extracellular fluid compartment. This did not occur. However, the interstitial fluid acts as a reservoir for the plasma, and the plasma volume changes only in extreme overhydration or dehydration (Cantarow and Trumper, 1949).

In the normal animal sodium and potassium are freely diffusible across the capillary membrane. One would therefore expect a fall in the plasma concentration of these ions if the extracellular fluid volume was increased by water alone. Since no change in the concentration of these ions was noted, it seems likely that the movement of fluid from the intracellular to the extracellular compartment was accompanied by diffusion of sodium and potassium out of the cells. Sodium as well as potassium are now known to be present within the cell.

In addition to the consistent increase in the thiocyanate, mannitol and bromide spaces produced by adrenal cortical extract there were occasional significant changes following the injection of desoxycorticosterone acetate and adrenocorticotropic hormone. Since no change was produced in a majority of the experiments, these occasional changes are not easy to explain. These values may represent technical errors or normal variation, but this

does not seem probable. It seems likely that there were imperceptible changes in the state of hydration or the hormonal balance of the animal. It is well known that many hormones react differently under different conditions (Anderson, 1948).

The transfer of fluid from the intracellular to the extracellular fluid compartment was brought about by whole adrenal cortical extract, but not by desoxycorticosterone acetate, the steroid most active in water and electrolyte metabolism. Wells and Kendall (1940) estimated that 90 percent of the water and electrolyte regulating activity of whole adrenal cortical extract remains in the amorphous fraction. It is possible that the internal rearrangement of the body fluids described in this paper may be due to the amorphous fraction.

SUMMARY

The acute (four hour) effect of adrenal cortical extract, desoxycorticosterone acetate and adrenocorticotropic hormone on the fluid distribution of normal dogs was measured. The plasma volume was measured by the distribution of T-1824 dye; the extracellular fluid compartment by the distribution of thiocyanate, mannitol and bromide; the total body water by the distribution of deuterium oxide. The reliability of the changes in the thiocyanate, mannitol and bromide spaces as a measure of changes in the extracellular fluid volume is discussed. The thiocyanate, mannitol and bromide spaces showed a consistent increase in volume after adrenal cortical extract injection. Desoxycorticosterone acetate and adrenocorticotropic hormone produced no consistent change. The plasma volume and total body water were unchanged by all hormones tested.

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ENHANCEMENT OF ADRENOCORTICOTROPIC HORMONE ACTIVITY BY ALUM IN NORMAL 21-DAY OLD MALE RATS

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THE bioassay of adrenocorticotropic hormone (ACTH) based on the increment of adrenal weight in normal 21–23 day old male rats was first suggested by Moon (1937). The general use of such animals has not had widespread acceptance for at least two reasons: (a) the presence of the pituitary in the animal may lead to variable and non-specific results; and (b) the method requires a relatively large dose of the hormone for any significant response. It seemed probable that the sensitivity of the Moon test could be enhanced by injections of a hormone-alum preparation, and the following experiments were carried out to test this point.

EXPERIMENTAL

The adrenocorticotropic hormone used in these experiments was a highly purified protein preparation isolated from fresh sheep pituitary glands by a modified method of Li, Evans and Simpson (1943). The pitressin-free adrenocorticotropic peptide mixture was obtained by HCl hydrolysis of the ACTH protein (Li, 1951). The alum-hormone solution was prepared by a procedure according to Kabat and Mayer (1948) by adding 5.0 cc. of 1% alum solution (potassium aluminum sulfate) to 100 cc. of a 0.15% ACTH solution. The solution was adjusted to maximal turbidity, usually about pH 4.7–4.8, by the slow addition of 0.1 *N* NaOH. When smaller volumes of alum precipitated solutions were prepared, a constant proportion of hormone to alum weight was maintained. Control solutions were made up of saline containing the same amount of alum as the alum-hormone preparations. The resulting suspension was shaken well before any injections were made. Twenty-one to twenty-three day old male rats of the Long-Evans strain were used in all experiments. Injections were always made subcutaneously on the backs of the animals.

The following experiments using the ACTH protein and peptide were carried out. A total hormone dose of 5.0 mg. per rat was employed in all cases:

(A) 9 injections of 0.5 to 0.6 mg. each were made during a period of 72 hours (3 injections per day) and the animals were autopsied 18 hours after the last injection.

(B) 4 injections of 1.25 mg. each were made during a period of 72 hours (1 injection per day) and the animals were autopsied 18 hours after the last injection.

(C) 1 injection of 5.0 mg. was made and the animals were autopsied 3 days (72 hours) later.

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(D) 2 injections of 2.5 mg. each were made 24 hours apart and the animals were autopsied 2 days (48 hours) after the last injection.

(E) This experiment, in which the acid peptide was employed, was conducted in the same way as Experiment B above.

The effects on adrenal and thymic weights were determined in these experiments. The resulting data were analyzed statistically by the method of Fisher (1950). Each of the experiments from B through E (Table 1) represents data pooled from two separate experiments; and Experiment A comprises data from three separate experiments.

RESULTS AND DISCUSSION

As Table 1 reveals, a total dose of 5.0 mg of the protein solution administered in 4 injections over a 3-day period (Experiment B) caused an insignificant change ($p > 0.1$) in the adrenal weight of 21–23 day old male

TABLE 1. BIOASSAY OF ALUM-TREATED ACTH PROTEIN AND PEPTIDE IN NORMAL 21–23 DAY OLD MALE RATS

Experiment	No. of injections	Autopsy+ time	Preparation	No. of rats	Body weight***		Adrenal	Thymus
					Initial	Final		
		hour			gm.	gm.	mg.	mg.
A	9	18	Control	19	53	63	17.7 ± 0.7*	130.1 ± 6.1
			Protein	25	53	64	24.7 ± 0.8 (<0.001)**	57.8 ± 4.5 (<0.001)
			Al-prot++	18	52	56	27.2 ± 1.4 (<0.001)	25.9 ± 1.8 (∨0.001)
B	4	18	Control	12	56	66	18.2 ± 0.7	153 ± 10.6
			Protein	12	56	62	20.0 ± 0.8 (>0.1)	90 ± 5.1 (<0.001)
			Al-prot	12	55	62	24.8 ± 1.0 (<0.001)	46 ± 4.4 (<0.001)
C	1	72	Control	12	56	67	17.7 ± 0.6	155 ± 9.8
			Protein	12	56	66	17.8 ± 0.7	140 ± 13.9 (>0.1)
			Al-prot	12	57	66	19.5 ± 0.6 (<0.05)	97 ± 12.4 (<0.001)
D	2	48	Control	12	54	65	16.4 ± 0.6	158 ± 9.5
			Protein	12	55	64	17.9 ± 0.5	110 ± 6.4 (<0.001)
			Al-prot	12	54	62	21.1 ± 0.7 (<0.001)	57 ± 7.9 (<0.001)
E	4	18	Control	12	54	66	19.6 ± 0.5	185 ± 8.3
			Peptide	13	54	63	20.5 ± 0.9	150 ± 7.5 (<0.001)
			Al-pep+++	13	54	63	21.4 ± 0.9 (>0.05)	98 ± 8.1 (<0.001)

A total dose of 5.0 mg. per animal was used in all experiments.
* Mean ± standard error.
** Fisher's p values, as compared with the control.
*** Initial body weight was taken just prior to the first hormone injection.
\+ Represents number of hours after the last hormone injection.
++ Alum-treated protein.
+++ Alum-treated peptide.

rats. On the other hand, when the same amount of hormone was injected as an alum-protein preparation, the adrenal weight manifested a statistically significant increase, rising from an average of 18.2 to 24.8 mg.

It is of interest to note in Experiment B that the injection of hormone protein alone effected a highly significant ($p<0.001$) reduction of thymus weight, in spite of the absence of a significant change in the adrenal weight. The same dosage of the alum-precipitated protein induced a further significant reduction of thymus weight, lowering the average from 90 mg to 46 mg. It is apparent, on the basis of the thymic involution observed, that there was adequate stimulation of the adrenals effected by the dose of ACTH protein which was employed. It is indicated by this and other experiments discussed in this paper that the increment of adrenal weight is not as sensitive an index of ACTH activity as is thymic involution.

The increment of adrenocorticotropic activity of ACTH protein by the alum treatment when given in one injection per day (Experiment B) reaches a level which compares favorably with that attained by dividing the same daily dose of the non-treated protein into 3 injections: i.e., a total of 9 injections in 3 days (Experiment A). This enhancement of ACTH potency can be seen from both adrenal weight increase and thymic involution. It appears reasonable, therefore, to assume that the enhancing effect of the alum on hormone activity is due to a delay in the absorption of the hormone. An interesting observation from Experiment A is that when the total dose of 5.0 mg is given in as many as 9 injections the superiority of the alum-treated protein as compared to the protein alone, is only barely evident from its effect on adrenal weight—inducing an increase of approximately 55% and 40% respectively, as compared with the controls. This phenomenon is probably due to the fact that when so many injections are made, a relatively higher level of circulating hormone is maintained, so that the advantage which can be obtained by delayed absorption does not become apparent. On the other hand, it may be noted that the thymus did reflect the enhanced effectiveness of the alum-hormone, the average weight being reduced to approximately one-half that of the non-alum treated hormone group.

In order to elucidate further this hypothesis of delayed absorption, experiments were performed to determine what relative differences in effect on adrenal and thymus weight would result, if only one or two injections were given to the animals and a relatively longer period allowed to elapse before autopsy. It may be seen in Table 1 (Experiment C) that no significant change in adrenal weight was evident in either the protein or alum-protein groups when a single dose of 5.0 mg was injected and autopsy was performed 72 hours later. However, the thymus weight was significantly reduced in the alum-protein treated animals from an average weight of 155

mg for the controls, down to 97 mg ($p < 0.001$), suggesting that the adrenal-stimulating activity may nevertheless have been present at the end of the 3 days after the single injection of the alum preparation.

When the total dose of 5.0 mg was divided into 2 injections given 24 hours apart and the animals were autopsied 48 hours after the last injection (Experiment D), no significant increase of adrenal weight was noted in the protein-treated group, while a significant increase ($p = < 0.001$) was evident in the alum-protein treated animals. There was a marked involution of thymus in both groups, while a much greater thymic involution was again revealed in the alum-protein treated group, with a decrease from an average of 158 mg for the controls to 110 mg and 57 mg respectively for the protein and alum-protein treated groups. Hence the suggestion is reinforced by the above observations, that alum does delay the absorption of ACTH protein, thereby prolonging the duration of the hormone's action.

The results obtained with the ACTH acid peptide are summarized under Experiment E of Table 1. It should be noted that in this case no significant change in adrenal weight was observed in either the peptide or the alum-peptide injected groups. However, the notable result was that the alum-peptide caused a marked involution of the thymus, amounting to approximately 50%, whereas the peptide itself reduced only slightly the thymus weight.

The apparent lack of effect of the ACTH acid peptide on the maintenance of adrenal weight in hypophysectomized rats, despite its high ascorbic-acid depleting activity, has recently been reported by Reinhardt and Li (1951). The present experiments employing normal rats show further that the ACTH acid peptide is relatively poor in its ability to cause an increase in adrenal weight.

SUMMARY

The alum precipitated preparation of highly purified ACTH protein has been investigated with respect to its ability to cause adrenal hypertrophy and thymus involution in 21–23 day old normal male rats. It was found that the adrenocorticotropic activity of the hormone was definitely enhanced by alum treatment, presumably through the effect of delayed absorption. When the ACTH acid peptide was used, neither the alum-peptide preparation nor the peptide itself affected significantly the adrenal weight; but a marked reduction of thymus weight was manifested in the alum-peptide treated animals, whereas only a slight reduction occurred in the animals injected with peptide alone.

It was consistently observed in all experiments that the involution of the thymus gland was a more sensitive index of the activity of the ACTH protein or peptide than the increment of adrenal weight.

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THE EFFECTS OF THYROID AND OF CHOLINE AND INOSITOL ON CHOLESTEROL DISTRIBUTION IN RATS FED A HIGH CHOLESTEROL DIET[1]

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IT WAS observed recently in rats fed a diet containing 1 per cent cholesterol and 0.5 per cent bile salt, that thyroid did not prevent a significant deposition of the sterol in the liver, although the plasma cholesterol content was reduced practically to normal levels (Marx, *et al.*, 1950). The possibility was considered that in the hyperthyroid state the requirement for lipotropic substances is increased. In order to study this interrelationship between thyroid and lipotropic agents further, the effects of choline and inositol on cholesterol and lipid distribution were investigated in normal and hyperthyroid rats fed a diet high in cholesterol and fat.

METHODS

Female rats (University of Southern California strain) 27 to 31 days old and weighing about 60 gms., were divided into seven groups. All animals were fed, *ad libitum*, a purified diet containing 20 per cent fat, 18 per cent casein, and 0.25 per cent bile salt.[2] The experimental groups were supplemented with the following components, either alone, or in combination: (a) 2 per cent cholesterol,[3] (b) 0.4 per cent thyroid,[4] and (c) choline and

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[2] Purified Diet: Salt mix, 4.5%; Casein, 18%; Wesson oil, 20%; Celluflour, 4%; Sucrose, 27%; Corn starch, 25%; Bile salt, 0.25% (dried extract of ox bile, USP). Vitamins per kg. of diet: Thiamine, 50 mg.; Riboflavin, 20 mg.; Pyridoxine, 20 mg.; Ca pantothenate, 60 mg.; Nicotinic acid, 60 mg.; Folic acid, 10 mg.; PAB, 200 mg.; 2-Methyl naphthoquinone, 5 mg. In addition the following were given once weekly orally (by syringe): 350 I.U. vitamin A, 35 I.U. vitamin D and 0.66 μg. vitamin B_{12}.

[3] Cholesterin—Merck and Co., Rahway, N. J.

[4] Thyroid Powder—U.S.P.—Armour and Co., Chicago, Illinois.

inositol; the latter were each fed at a level of either 0.25 per cent or 1.0 per cent of the diet. The dietary supplements of the experimental groups are summarized in Table I.

Body weights were recorded weekly and, after an experimental period of seven weeks, the animals were autopsied (nembutal anesthesia). Blood was drawn from the vena cava. Liver, and in some instances lungs and adrenals, were excised as quickly as possible, minced with scissors, and extracted, using a Soxhlet apparatus, first with 95% alcohol and then with ether. Total lipid and cholesterol contents were then determined, the latter according to a modified Schoenheimer-Sperry-Chaney procedure (Nieft and Deuel, 1949). The aorta was taken for histological examination which was made as soon as possible, to avoid loss of cholesterol during storage in formalin (Marx *et al.*, 1950).

RESULTS

The patterns of the body weight gains were very similar in all experimental groups, and not different from those of normal rats. The thyroid feeding produced the symptoms typical of hyperthyroidism, such as an increased nervousness, an increased respiratory rate, a shaggy coat, an increased food and water intake and fecal excretion, but the level of the hormone given was not high enough to be severely toxic. For the groups not supplemented with cholesterol, the results are summarized in Table II.

TABLE I. EXPERIMENTAL GROUPS

Group	Dietary Supplements*				Number of rats
	2% Cholesterol	0.4% Thyroid	Choline + Inositol		
			.25% Each	1.0% Each	
I	−	−	−	−	7
II	+	−	−	−	6
III	−	+	−	−	7
IV	−	−	+	−	9
V	+	+	−	−	7
VIa	+	−	+	−	8
VIb	+	−	−	+	7
VIIa	+	+	+	−	9
VIIb	+	+	−	+	5

* + indicates: present in diet.
− indicates: absent from diet.

TABLE II. EFFECTS OF THYROID AND OF CHOLINE PLUS INOSITOL IN RATS FED A PURIFIED DIET CONTAINING 20% FAT

Group	Dietary supplement	Plasma cholesterol mg. %	Liver		
			Cholesterol %	Total lipids %	Weight, % of body weight
0*	None	60 ± 3	.21 ± .02	5.9 ± .4	3.3 ± .1
I	None	60 ± 4	1.12 ± .12	21.5 ± 1.3	5.5 ± .3
III	.4% Thyroid	65 ± 7	.34 ± .01	9.5 ± 1.3	5.5 ± .1
IV	.25% of each Choline and Inositol	80 ± 8	.30 ± .02	6.7 ± .5	4.4 ± .1

* Data for a control group of 17 rats fed Purina Chow (Marx *et al.*, 1951).

The results indicate that the purified high fat diet lacked sufficient quantities of lipotropic substances, in spite of a casein content of 18 per cent, to prevent a significant lipid and cholesterol deposition in the liver. The addition of either thyroid or choline and inositol to the basic diet reduced these values towards normal levels, though the hepatic lipid content in the hyperthyroid group remained significantly elevated.

The effects of thyroid and of choline and inositol in rats fed the high cholesterol diet are summarized in Table III. The animals receiving cholesterol and bile salt alone showed a moderate increase in plasma cholesterol but an extreme accumulation of hepatic sterol and total lipids; the liver weight also was markedly elevated.

TABLE III. EFFECTS OF THYROID AND OF CHOLINE PLUS INOSITOL ON CHOLESTEROL DISTRIBUTION AND LIVER LIPIDS IN RATS FED 2% CHOLESTEROL AND .25% BILE SALT

Group	Dietary supplement*	Plasma cholesterol mg. %	Liver		
			Cholesterol %	Total lipids %	Weight, % of body weight
II	None	107 ± 7	10.0 ± .8	37 ± 2	7.5 ± 2.6
V	Thyroid	74 ± 8	5.4 ± .5	29 ± 3	9.5 ± 1.2
VIa	.25% Choline + Inositol	253 ± 26	7.6 ± .8	26 ± 1	5.9 ± .2
VIb	1.0% Choline + Inositol	171 ± 27	4.3 ± .7	19 ± 1	5.0 ± .5
VIIa	Thyroid + .25% Choline + Inositol	165 ± 24	5.9 ± .6	21 ± 1	7.5 ± .2
VIIb	Thyroid + 1.0% Choline + Inositol	131†	2.9 ± .6	14 ± 1	5.9 ± .1

* All groups received cholesterol plus bile salt.
† Plasma from 5 animals pooled.

Thyroid feeding caused a significant drop in plasma and liver cholesterol and in liver lipids; the former approached normal levels, but the liver values were still markedly elevated. In spite of this reduction in hepatic lipid fractions, thyroid hormone produced a further rise in liver weight.

As a consequence of the administration of choline and inositol the plasma cholesterol was markedly elevated; this effect was greater with the lower dose of the lipotropic factors. In the liver, the values for the sterol and total lipid contents, and for the weight of this organ were significantly reduced; the hepatic response was correlated with the dose level.

The effects of a supplementation with thyroid together with choline and inositol were roughly additive excepting the action of thyroid on liver cholesterol which was not significantly modified by the lower dose level of the lipotropic substances. It is noted that under the conditions of these experiments even a combination of the endocrine and dietary lipotropic agents was not sufficient to prevent a still significant accumulation of cholesterol and lipid in the liver.

In all groups, the aorta was macroscopically normal. Histological exam-

ination revealed that administration of thyroid to rats fed cholesterol and bile salt had a tendency to cause a deposition of small amounts of cholesterol crystals diffusely scattered through the aortic endothelium, and a swelling of the latter. Choline and inositol at a level of 0.25 per cent did not significantly influence this phenomenon.

DISCUSSION

The feeding of a high cholesterol diet produced results qualitatively similar to those observed previously by a number of investigators (reviews quoted by Marx *et al.*, 1951); under the conditions of the present work, the fat and cholesterol accumulation in the liver was remarkable, in particular, if the pronounced increase in liver weight is taken into consideration.

The relation of the thyroid hormone to the plasma cholesterol level, again confirmed by the present work, is too well established to warrant further discussion (reviewed, e.g., in Peters and Van Slyke, 1946). The influence of thyroid on the sterol content of other tissues does not always follow the pattern of an inverse relationship. According to a number of investigators, thyroid powder or thyroxin elicited an increase in the cholesterol content of various organs, in particular of the liver (Sakai, 1929; Parhon and Werner, 1939; Pasternak and Page, 1934), of the skin (Abelin, 1940), and of the whole carcass (Pasternak and Page, 1935; Fleischmann and Shumacker, 1942; Schettler, 1950). On the other hand, in cases of an accumulation of cholesterol in tissues, in particular in the liver and aorta, produced by dietary or endocrine mechanisms, thyroid was reported to cause a mobilization of the deposited sterol (Murata and Kataoka, 1917; Turner, 1933; Handler, 1948; Dauber *et al.*, 1949; Stamler, Silber *et al.*, 1950). The results described above confirm the latter observations. It is interesting that the thyroid hormone exhibited a significant lipotropic activity, as far as hepatic total lipids and sterol were concerned, in spite of a relative deficiency of dietary lipotropic substances. The thyroid treatment was unable, however, to prevent a still severe lipid and cholesterol accumulation in the livers of the cholesterol-fed animals, in line with earlier observations (Marx *et al.*, 1950), and with results obtained by Forbes (1944) who found that thyroxine did not prevent a deposition of cholesterol in the liver, unless the choline intake was adequate. On the other hand, Handler (1948) found that thyroid hormone mobilized hepatic cholesterol even in choline-deficient rats.

The recent observation that thyroid administration elicited the appearance of a diffuse scattering of cholesterol crystals in the aortic wall of cholesterol-fed rats (Marx *et al.*, 1950), was confirmed by the results presented above. It is to be noted that this type of diffuse cholesterol deposition is different from the focal cholesterol accumulation in atheromatosis,

and that it was neither correlated with the plasma cholesterol level, nor modified by dietary choline and inositol. It is not known whether the observed sterol deposition occurred as a result of a transfer of the sterol from plasma into tissues, a mechanism postulated by Fleischmann and Schumacker (1942); as a consequence of an increased rate of endogenous cholesterol synthesis which appears possible in view of findings by Karp and Stetten (1949) and Siperstein *et al.* (1951); or whether another mechanism was responsible.

The results obtained with the administration of the lipotropic substances seem to indicate that, in rats fed a low cholesterol diet, the plasma cholesterol is not significantly modified, in spite of a pronounced effect on the liver sterol and total lipid values, confirming previous observations by Handler (1948). In the groups fed the high cholesterol diet, however, choline and inositol caused a remarkable increase in plasma cholesterol, more pronounced at the lower level of administration. The reports in the literature related to the action on the plasma sterol of lipotropic substances in conditions of hypercholesterolemia are contradictory. A number of investigators observed a reduction of plasma cholesterol as a consequence of the administration of choline and/or inositol (Herrmann, 1947; Leinwand and Moore, 1949; Felch and Dotti, 1949). Others found that the feeding of choline alone, or in combination with inositol did not modify plasma sterol levels, neither in cholesterol-fed normal animals (Himsworth, 1938; Firstbrook, 1950), nor in hypothyroid (Davidson *et al.*, 1950) nor in thyroidectomized-hypophysectomized dogs (Entenmann *et al.*, 1948), nor in partially hepatectomized rats (Byers *et al.*, 1951). It is realized that it is difficult to compare the present results with these earlier findings, since the experimental conditions and the animal species used were different. The authors are not aware of any reports dealing with similar observations in the normal rat; the data described above are in line, however, with results of Stamler, Bolene *et al.* (1950) who demonstrated that in cholesterol-fed chickens choline and inositol significantly aggravated the hyperlipemia and hypercholesterolemia produced by a high cholesterol intake.

The question whether thyroid hormone is capable of mobilizing liver total lipids and cholesterol in the absence of lipotropic substances, cannot be answered unequivocally by the present work, since the basic diet was not completely free of, although deficient in, lipotropic factors. The results indicate, however, that the action of the thyroid hormone on the mobilization of hepatic total lipids and cholesterol is significantly enhanced by choline and inositol, and vice versa, in agreement with observations by Forbes (1944) and Handler (1948).

The phenomenon of lipotropism is too well known to require any further discussion (reviewed, e.g., by Best and Lucas, 1943). It is interesting that

choline and inositol did not modify the thyroid-induced cholesterol deposition in the aorta of the cholesterol-fed rats, although exhibiting a marked lipotropic activity with regard to the liver of the same animals. A number of investigators have studied the effects of choline, and, in some cases, of inositol, on the cholesterol content and atheromatosis of the aorta of cholesterol-fed animals (Steiner, 1938; Morrison and Rossi, 1948; Broun *et al.*, 1949; Stamler, Bolene *et al.*, 1950, who quote most of the earlier papers pertaining to this subject; Firstbook, 1950; Davidson *et al.*, 1950) but the results are contradictory.

SUMMARY

Plasma and liver cholesterol and liver total lipids were determined, and the aorta was examined histologically, in rats fed a purified diet containing 20 per cent fat, 18 per cent casein and 0.25 per cent bile salt, and supplemented with the following components, either alone, or in combination: (a) 2 per cent cholesterol; (b) 0.4 per cent thyroid USP; (c) choline and inositol, either 0.25 per cent or 1.0 per cent each.

It was observed that thyroid diminished significantly the moderate hypercholesterolemia and severe cholesterol and lipid accumulation in the liver of cholesterol-fed rats.

Choline plus inositol produced a marked hypercholesterolemia, and, at the same time, a significant drop in hepatic sterol and lipoid values in the group fed the high cholesterol diet.

When the endocrine and dietary agents were combined, their effects on the plasma sterol were essentially additive; they enhanced each other's effects, as far as the mobilization of hepatic cholesterol and total lipid was concerned, except at the lower level of the lipotropic substances which did not significantly modify the action of the thyroid hormone.

The aorta was macroscopically normal in all groups. In the cholesterol fed rats, thyroid caused the appearance of microscopic cholesterol crystals scattered diffusely through the aortic wall; 0.25 choline and inositol did not significantly influence this phenomenon.

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A GLYCOSTATIC EFFECT OF TESTOSTERONE ON THE PERINEAL MUSCLES OF THE RAT[1]

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THE question as to whether glycogen storage (glycopexis) in skeletal muscle is influenced by the amount of circulating androgens in the male has not been satisfactorily answered. Schuman (1940a, b) and Borgatti (1947) reported that testosterone increased and castration decreased the storage of glycogen in skeletal muscle, but on the other hand, negative results were obtained by Gaunt, et al. (1939), Cahane (1940), Griffiths (1941), McCullagh and Lewis (1942), and Winter and Thompson (1944). One of the leg muscles was used as test material in the above experiments. The fact that the striated muscles of the perineal complex of the male rat decrease in size following castration and increase in size under the influence of male hormone (Wainman and Shipounoff, 1941) suggests that these muscles might show definite alterations in glycogen concentration relative to androgen stimulation. This paper will report the changes which occur in the glycogen concentration of these muscles following castration and testosterone therapy and will show that the effect of testosterone on the glycogen levels of these muscles is independent of the pituitary.

METHODS

Adult male rats of the Long-Evans strain were used; there body weights and ages were quite uniform within a given experiment. Castration and hypophysectomy were performed under ether anesthesia and at the end of the experiments, the completeness of each operation was verified. A standard diet (calf meal ration) was fed *ad libitum* and, in addition, a 15% glucose solution was given as a free choice to all hypophysectomized rats. Testosterone propionate[2] in peanut oil was injected; the controls received only peanut oil.

The rats were given no food for 24 hours before removing the muscles, always between 8–9 A.M. During the fast, they had access to water. The animals were anesthetized by an intraperitoneal injection of Nembutal (6 mg. per 100 gm. body weight) and the perineal muscles dissected out as previously described (Leonard, 1950). The muscle samples, ranging from 200–400 mg., were weighed quickly on a torsion balance and placed in a test tube containing 2 ml. of 30% KOH. The tubes were placed immediately

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[2] The testosterone propionate used in this study was furnished through the generosity of The Schering Corporation, Bloomfield, New Jersey.

in a boiling water bath. Since some loss of the labile glycogen in the muscles must have occurred during the prolonged dissection, tared tubes with KOH were not considered necessary. For comparative purposes this method proved satisfactory.

For the determination of glycogen as glucose, the methods of Good, Kramer and Somogyi (1933), and Shaffer and Somogyi (1933) were employed.

EXPERIMENTAL

Effect of castration. In the course of the investigation it was discovered that the length of time of castration markedly influenced the results. Groups of rats were castrated for varying periods of time, from 1 to 96 days, and the perineal muscles assayed for glycogen. The controls of the rats castrated for 1 and 2 days were subjected to mock operations so that the possible effects of trauma would be obviated. The results are presented in Table 1.

TABLE 1. PERINEAL MUSCLE GLYCOGEN IN NORMAL AND CASTRATED RATS*

Period of castration (days)	Body weight (gm.)		Glycogen (mg./100 gm.)	
	Castrate	Control	Castrate	Control
1	(9) 319	(9) 316	*291 ± 13*	341 ± 16
2	(8) 328	(9) 334	*247 ± 5*	337 ± 17
7	(12) 282	(13) 280	*268 ± 17*	394 ± 17
15	(6) 308	(6) 315	337 ± 12	366 ± 25
24–26	(8) 280	(8) 315	413 ± 40	420 ± 27
60†	(6) 293	(4) 335	422 ± 13	431 ± 7
96	(4) 348	(4) 337	391 ± 40	356 ± 23

* Numbers in ( ) refer to number of rats.
Results are given with the standard error of the mean. Numbers in italics are significantly different from their respective controls.
† Rats were not starved before autopsy.

It was noted that castration for periods of 1, 2, and 7 days resulted in a significant decrease in muscle glycogen. The percentage decrease was 15%, 27% and 32% respectively for these periods. Statistically these differences are significant, the probabilities that the results were not due to chance alone are $P > .02$ for the 1-day castrates and $P < .01$ for the other two groups.

When a period of 15–96 days after castration elapsed, no difference was found in the muscle glycogen of the experimental and control rats in spite of the fact that these muscles, by 30 days after gonadectomy, had regressed to approximately a third of their normal size. No care was taken to dissect all the muscle tissue from the perineal complex since an adequate sample only was necessary for analyses before glycogenolysis had progressed too far. Thus the total actual weight of the muscles in the experimental and control animals was not obtained but the estimation of regression was noted from other data.

Effect of testosterone treatment. Preliminary experiments indicated that

injections of 1 mg. of testosterone propionate per day for 3 days caused a marked increase in the muscle glycogen of the perineal complex. Rats which were castrated for periods of 42–50 days and injected with a total dosage of 3 mg. of male hormone showed a 116% increase in muscle glycogen (see Table 2). Also rats which were castrated for 7 days and injected for 3 days had levels of muscle glycogen considerably higher than their respective controls which were, in this instance, intact rats injected with oil. Finally, a group of intact males were given the same hormone treatment as above and the muscle of these rats also showed a significant increase in glycogen over that of their normal controls.

TABLE 2. PERINEAL MUSCLE GLYCOGEN IN NORMAL AND CASTRATED RATS TREATED WITH TESTOSTERONE

Experiment*	Body weight (gm.)		Glycogen (mg./100 gm.)	
	Treated	Control	Treated	Control
Normal males	(5) 302	(5) 302	721 ± 43	466 ± 24
Castrated 42–50 days	(10) 326	(10) 321	814 ± 25	377 ± 25
Castrated 7 days, controls intact males	(4) 337	(4) 339	691 ± 10	381 ± 22
Castrated 4 days, 1 injection of 1 mg. T.p. for 24 hours	(4) 275	(4) 278	535 ± 21	255 ± 11

* Rats received 1 mg. testosterone propionate per day for 3 days before autopsy, except in the last experiment.

To obtain some concept of the rapidity with which testosterone exerted its glycopexic effect on the perineal muscles, rats which had been castrated for 4 days were given a single injection of 1 mg. of the hormone and were examined 24 hours later. The results showed that the male hormone doubled the amount of glycogen in the muscles over that in the controls (Table 2).

Hypophysectomized rats. It is well known from the extensive works of Dr. Jane Russell and co-workers that pituitary extracts (now believed to be the growth hormone), prevent the loss of glycogen from skeletal muscle which occurs in a hypophysectomized rat following a 24 hour fast (Russell, 1938; Russell and Wilhelmi, 1950). This effect of the pituitary extract (glycostatic effect) is qualitatively independent of some other endocrine secretions known to be involved in carbohydrate metabolism (Bennett and Perkins, 1945). Furthermore, the effect is specific for striated muscle (gastrocnemius, diaphragm, heart) and does not involve the store of liver glycogen (Illingworth and Russell, 1951). These findings suggested the possibility that testosterone might have exerted its effect *via* the pituitary and to test this, hypophysectomized rats were employed.

In order to determine whether the perineal striated muscles retained or lost their glycogen supply after a fast, 12 adult male rats of uniform size were hypophysectomized. Six days later, half of the rats were starved for

24 hours and the perineal muscles and the thigh muscles of one leg, from all the animals, were assayed for glycogen. The average glycogen content of the the perineal muscles of 6 rats well fed was 356 ± 17 mg. per 100 gm. of muscle and that from 6 starved animals was 251 ± 15 mg. The thigh muscles averaged 478 ± 18 mg. and 215 ± 4 mg. respectively. This confirms Russell's work and show that the perineal muscles respond in a similar manner.

A group of rats was hypophysectomized and half of the group received testosterone propionate daily for 7 days. In another group, a lapse of 1 week after hypophysectomy intervened before hormone therapy was begun. In both experiments the glycogen content of the perineal muscles was markedly elevated in the treated rats when compared with that of the controls (Table 3).

TABLE 3. PERINEAL MUSCLE GLYCOGEN IN HYPOPHYSECTOMIZED RATS TREATED WITH TESTOSTERONE

Experiment	Body weight (gm.)		Glycogen (mg./100 gm.)	
	Treated	Control	Treated	Control
Inject. 13.7 mg. Testos. for 7 days, immediate treatment.	(8) 240	(7) 237	455 ± 28	217 ± 9
Inject. 7 mg. Testos. for 7 days, postponed treatment.	(5) 226	(5) 211	526 ± 24	328 ± 10
Inject. 3 mg. Testos. for 3 days, begin. 4 days after operation.	(4) 189	(5) 187	557 ± 40	262 ± 9

In order to obtain some information on the optimum period of injection to demonstrate the effect of testosterone propionate, 8 rats which were hypophysectomized for 1 week, were injected in pairs with 1 mg. of hormone per day for 1, 2, and 3 days. The animals were starved as before, and the perineal muscles assayed. The average muscle glycogen of the controls was 234 mg. per 100 gm. of muscle, and for the injected rats, 444 mg., 574 mg. and 685 mg. respectively, for the periods of treatment. A period of treatment for 3 days appeared adequate to demonstrate this effect of testosterone propionate as the results of the last experiment in Table 3 indicate.

Although Bennett and Perkins (1945) had observed that the adrenal gland played no essential part in the glycostatic effect of the pituitary hormone, the possibility remained that testosterone could have exerted an effect on carbohydrate storage in the treated animal. This is based on the observations of Leonard (1944) and Leatham (1944) that testosterone slows the rate of structural degeneration of the adrenal cortex of hypophysectomized rats. The adrenal cortex, if it were functional, would continue production of the hormones capable of maintaining storage of liver glycogen. To test this, the livers of the hypophysectomized rats in the first group listed in Table 3 were analyzed for glycogen. It was found that the

livers of the testosterone-treated rats contained 43 ±4 mg. of glycogen per 100 gm. of liver and the controls 44 ±4 mg.

Incidental to this study, glycogen determinations were made on the testes of those rats which were hypophysectomized for 1 week before injections of testosterone propionate were begun (listed in Table 3). Although the average weights of the testes of the treated and control rats were 1.644 gm. and 1.053 gm. respectively, the glycogen concentrations were 23 ±5 mg. per cent and 25 ±1 mg. per cent. Thus neither the liver nor testes were altered in their glycogen content.

DISCUSSION

The results of the first experiment showed that castration produced a rapid but transitory decrease in the glycogen concentration of the perineal muscles in the rat. On the other hand, Schuman (1940a) observed a decrease in the glycogen concentration of the leg muscles of the rabbit following castration which became evident only after several weeks had elapsed. Although histologically the muscles are similar, the difference in response to castration may be due to a species difference or to some special quality of the perineal muscles related to their behavior as a secondary sexual character (Wainman and Shipounoff, 1941). No studies were made on the effect of castration on the leg muscles of the rat at this time because of the possibility that the prolonged manipulation of the animal in removing the perineal muscles might have permitted glycolysis in the other muscles to occur.

The injection of testosterone propionate increased the glycogen content of the perineal muscles quite definitely in normal, castrated and hypophysectomized rats. These results agree with those of Schuman (1940a) which were reported for the leg muscles of castrated rabbits given similar treatment. It should be noted that remarkable powers of growth are exhibited by the perineal muscles under the influence of long continued injections of the hormone and it is possible to produce over-sized muscles by this treatment (unpublished data). Thus it seems that the increase in the concentration of glycogen is an index of renewed growth activity in these muscles. The deposition of glycogen in skeletal muscle induced by injections of testosterone is not limited to the perineal muscles because several preliminary experiments have indicated a significant increase in glycogen in the leg muscles of treated hypophysectomized rats. Kochakian (1946) has reviewed the biochemical evidence on the anabolic property of testosterone and from this it is suggested that the increase in glycogen in muscle may be indicative of the mechanism by which the hormone exerts its growth stimulating effect.

The application of the term "glycostatic effect" to testosterone seems

justified with respect to its behavior towards glycogen in the perineal muscles. The maintenance of a high concentration of glycogen in the leg muscle of fasted hypophysectomized rats injected with the pituitary hormone (Russell, 1938) corresponds in effect to that of testosterone acting on the perineal muscles. Furthermore, the male hormone acts independently of the pituitary hormones, at least on a qualitative basis. Possibly there is some quantitative relationship between the pituitary hormone and testosterone in regulating glycogen concentration in these muscles. The highest concentration of glycogen obtained in these experiments was in the muscles of those animals having an intact pituitary and injected with testosterone propionate.

The liver glycogen of the hypophysectomized rats was not effected by the injections of testosterone propionate. Although it has been shown that morphologically, the adrenal cortex is partially maintained by testosterone (Leatham, 1944; Leonard, 1944), the evidence indicates that cortical hormones are not produced in sufficient quantities if at all, to stimulate glycogen storage in the liver.

SUMMARY

It was shown that castration in adult male rats for periods of 1–7 days was accompanied by a significant decrease in glycogen concentration in the striated muscles of the perineal complex. After periods of castration for 15–96 days, no difference in the glycogen concentration was found. Injections of testosterone propionate into normal and castrated rats resulted in a marked increase in glycogen in these muscles. The effect of the hormone can be demonstrated within 24 hours after administration.

Since testosterone propionate prevented the glycogen level from declining during a fast in the hypophysectomized animal, the effect is a direct one and testosterone can be said to exhibit "glycostatic" properties under these conditions. The concentration of glycogen in the liver and testes was not effected by testosterone propionate in the hypophysectomized rat.

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WATER EXCRETION IN HYPOPHYSECTOMIZED RATS BEFORE AND AFTER ADRENALECTOMY[1,2]

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THE hypophysectomized rat, as well as the adrenalectomized rat, displays an impaired ability to excrete a test load of water (Walker, 1939; Chen and Geiling, 1943; Joseph, Schweizer, Ulmer and Gaunt 1944). Whether or not this impairment persists unchanged in the post-operative period is a matter of dispute; Walker (1939) found the rate of excretion of a water load by hypophysectomized rats to increase as the time after hypophysectomy increased, while Joseph et al. (1944) observed the excretory rate to remain essentially unchanged.

Since adrenalectomy in the hypophysectomized rat leads to the death of the animal shortly thereafter (Greep, 1950), the adrenal cortex must play some role vital to the continued survival of the animal, even in the absence of the adrenocorticotropic hormone of the anterior pituitary. Just what function it performs in the hypophysectomized animal is unknown. In this study the posible influence of the adrenal cortex on water metabolism in the hypophysectomized rat has been investigated.

MATERIALS AND METHODS

Adult female rats of the Hisaw strain, weighing from 175–200 grams, were used. Hypophysectomy was performed via the parapharyngeal route. Bilateral adrenalectomy was performed in a single-stage operation. Completeness of hypophysectomy was attested to by the development of cachexia and by target organ atrophy and verified by examination of the sella turcica under a binocular dissecting microscope. Completeness of adrenalectomy was established by the death of the animals within approximately twenty-one days after operation, this having been established as average maximal survival time for this size and strain of rat under our laboratory conditions.

To test any possible influence of diet on the excretory performance of the animals, they were divided into two groups: Group I, containing twelve rats, was fed Purina Laboratory Chow pellets; Group II, containing six rats, was offered the Chow pellets in a ground form and a synthetic diet (Ration 100), the latter being recommended by Shaw

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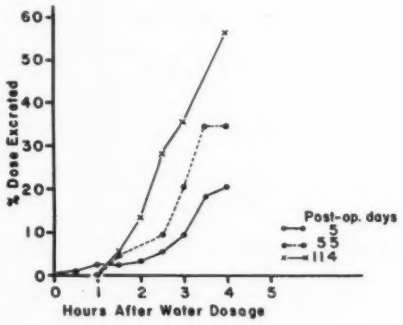
and Greep (1949) for hypophysectomized rats. As a whole, Group II showed no marked preference for either diet.

The water loading tests were conducted as follows: at the end of a 16 to 18 hour fast, the animals received 5 cc. of tap water per 100 gms. of body weight by stomach tube, the water being at body temperature. The rats were then placed in individual metabolism cages, the funnels of which were equipped with feces traps. The urine drained into 25 cc. cylinders, graduated in cc., from which readings of urine volumes to the nearest 0.5 cc. were made at half-hour intervals. Food and water were withheld from the animals during the test period.

RESULTS

1. *Influence of the length of time after operation on the excretory ability of hypophysectomized rats*

The rate of excretion of a test load of water at varying times after hypophysectomy was measured and the results are shown in Fig. 1. A very



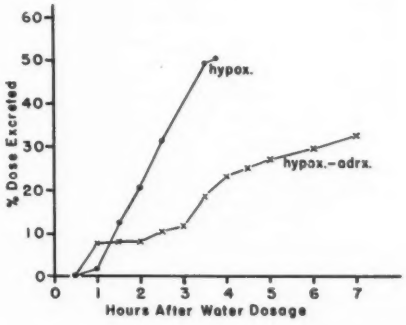


FIG. 1. Correlation between length of postoperative period and rate of excretion of a test load of water by hypophysectomized rats.

Fig. 2. Effect of adrenalectomy on rate of excretion of a test load of water by hypophysectomized rats (hypox. = hypophysectomized; adrx. = adrenalectomized).

definite improvement in excretory ability as the time after hypophysectomy increased can be noted, and was observed in both groups of rats. In this respect, these data confirm the findings of Walker. (1939); however, these animals failed, by a wide margin, to achieve the rapid excretory rates which he reports for hypophysectomized rats. Conversely, these data do not support the findings of Joseph et al. (1944). Moreover, the animals in this study displayed excretory rates much more rapid than they reported for their hypophysectomized rats.

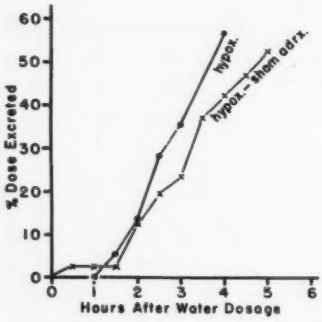
2. *Influence of diet on excretory ability of hypophysectomized rats*

A comparison of the excretory rates of Groups I and II in the hypophysectomized state, as shown in Figs. 2 and 3, reveals no significant differ-

ence. Thus while the synthetic diet (Ration 100) permits a longer survival of hypophysectomized rats and even some body growth (Shaw and Greep, 1949), it apparently has little effect on their ability to excrete a test load of water.

3. *Influence of adrenalectomy on ability of hypophysectomized rats to excrete a test load of water*

One hundred and eighty days after hypophysectomy, the response of the rats in Group I to a test load of water was determined. Eight days later they were adrenalectomized and the water loading test repeated three days



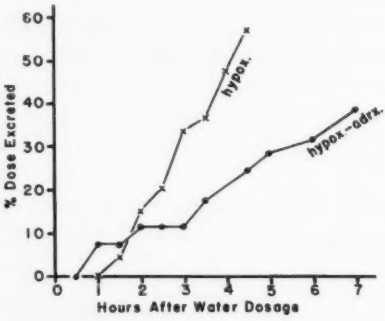


FIG. 3. Effect of sham adrenalectomy on rate of excretion of a test load of water by hypophysectomized rats.

FIG. 4. Effect of adrenalectomy on rate of excretion of a test load of water by hypophysectomized rats.

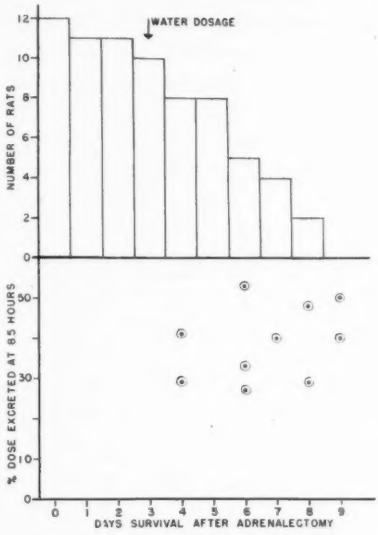
after this. On this latter occasion, only one animal, by marked inactivity and profuse salivation, showed signs that the water dose might be toxic.

Figure 2 shows the average excretory rate for the group before and after adrenalectomy. A marked decrease in the rate of excretion of a test load of water after this operation can be clearly seen.

Clear cut as these results are, certain control observations seemed essential to strengthen the validity of this finding.

To rule out possible adverse effects due to the operative trauma of adrenalectomy, the excretory rates of the rats in Group II were measured 114 days after hypophysectomy. Sham adrenalectomy was performed nine days later, and the water loading test repeated on the third post-operative day for this latter operation. The results, shown in Fig. 3, reveal no significant difference in excretory rates as a result of the sham operation. Rather the difference seen lies well within daily fluctuations in excretory performance of this size group of rats.

Since adrenalectomy inevitably results in the early death of the hypoph-



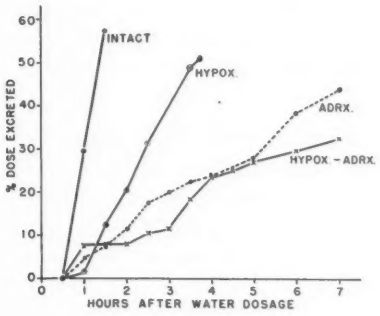


FIG. 5. Survival of hypophysectomized rats after adrenalectomy and correlation between survival after adrenalectomy and performance in the water-loading test.

FIG. 6. Rate of excretion of a test load of water by intact, adrenalectomized, hypophysectomized and hypophysectomized-adrenalectomized rats.

ysectomized rat, possible pre-terminal morbidity must be taken into account. Figure 5 shows that 50 per cent of Group I lived three days or more after the test; this finding tends to minimize any possible influence imminent death might have had on the results. By common experience morbidity preceding death due to adrenal insufficiency, has a rather sharp onset in the rat and occupies roughly a period of from twenty-four to thirty-six hours. Nor is there any demonstrable correlation between performance in the test situation and length of survival after adrenalectomy, as shown in Fig. 5.

In further confirmation of this finding with Group I, the rats in Group II were given water loads on the 196th day after hypophysectomy, their adrenals removed three days later and the test repeated after an additional three days. The results (Fig. 4) not only confirm those obtained from Group I, but a marked similarity in the actual excretion rates of the two groups after adrenalectomy can be seen.

To obtain a more composite picture of hypophyseal-adrenal interrelationships and influences on water metabolism, six intact rats of the same age, sex and strain were subjected to the water loading test along with ten adrenalectomized rats. The latter were tested on the twelfth post-operative day, having been supplied with 0.9% NaCl as drinking fluid until twenty-

four hours before the test. Fig 6 shows this composite picture. (The data for the hypophysectomized rat before and after adrenalectomy are those shown in Fig. 2). It can be seen that the long term hypophysectomized rat excretes a test load of water at a rate significantly greater than that of the adrenalectomized rat, but that adrenalectomy in the former animal reduces its excretory rate until it approximates that of the adrenalectomized animal.

DISCUSSION

Swann (1940) first suggested that the adrenal cortex might be capable of some function independent of the anterior pituitary, and Deane and Greep (1946) have pointed out that the zona glomerulosa of the adrenal cortex, as judged by histological and histochemical criteria, appears healthy after hypophysectomy in the rat and may even increase its mass. Since the hypophysectomized rat dies when adrenalectomized, one can hardly argue other than that the adrenal cortex does play a vital role in the physiology of the hypophyseoprivic rat. The results reported here offer support to this concept and permit a rough quantitation of cortical function after hypophysectomy in regard to the excretion of a test load of water.

The excretory rates for intact and adrenalectomized rats in this study agree favorably with those reported elsewhere; however, the rates of excretion of a water load by the hypophysectomized rats in this study are less than those reported by Walker (1939), and greater than those reported by Joseph et al. (1944). Differences in strain of rat, housing conditions or test conditions could contribute to this discrepancy. However, any possible role of diet is rendered unlikely by these data.

The finding that the long term hypophysectomized rat excretes a test load of water at a faster rather than the adrenalectomized rat is an interesting one. The qualifying phrase "long term" must be emphasized for a comparison of Figs. 1 and 6 shows that there is little difference between the excretory rates of a hypophysectomized rat five days after operation and an adrenalectomized rat. The improvement on the part of the hypophysectomized rat in handling a water load may be related to the growth of the zona glomerulosa after hypophysectomy, as noted by Deane and Greep (1946). The difference between the excretory rates of the hypophysectomized and the adrenalectomized rat could be explained on the basis of an agent, present in the adrenalectomized rat and absent in the hypophysectomized rat, which tends to retard urine flow. Gaunt, Birnie and Eversole (1949) note a fundamental difference between these two animals in regard to factors acting on water metabolism and suggest the possible role of the posterior pituitary as a deterrent to rapid water excretion.

If it is granted that adrenal cortical hormones promote rapid water diuresis and that the hormone of the posterior pituitary retards it, then

the following heuristic explanation could be offered for the excretory deficits seen in the hypophysectomized and adrenalectomized rat. Rapid water diuresis is retarded in the adrenalectomized rat due to the absence of the adrenal cortex and the presence of the posterior pituitary; it is retarded to a lesser extent in the hypophysectomized rat because of reduced adrenal cortical function, the absent posterior pituitary playing no role in this situation. The deficit may also be abetted by inadequate thyroid function in the latter case. Removal of the adrenals in the hypophysectomized rat then withdraws the last support to rapid water diuresis and a further deficit in the excretion of a water load is seen. The concept of posterior pituitary-adrenal cortical antagonism, so important to this explanation, was first offered by Silvette and Britton (1938) and is discussed in detail by Gaunt et al. (1949). The above, beyond doubt, is an over-simplified explanation of what appears to be a very complex situation.

SUMMARY

The rate of excretion of a test load of water before and after adrenalectomy was measured in two groups of hypophysectomized rats, each on a different diet.

With the diets employed, no significant difference in excretory rates between the two groups could be observed.

The hypophysectomized rat excretes a test load of water more slowly than an intact animal. This is further and markedly reduced following adrenalectomy, thereby indicating that the adrenal cortex does possess some degree of function even in the absence of the anterior pituitary. This further deficit in water excretion induced by adrenalectomy is apparently not due to operative trauma or pre-terminal morbidity.

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RESPONSE TO ALLOXAN MODIFIED BY GONADECTOMY

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A RECENT communication (Beach, Bradshaw and Blatherwick, 1951) demonstrated that female albino rats more frequently develop severe diabetes when injected with alloxan than do males. It was thought that this sex difference might depend upon the presence of the gonads and, consequently, an attempt was made to eliminate it by castration. Contrary to expectations, gonadectomy failed to eliminate the hypersusceptibility of females to alloxan, a larger proportion of castrate females than castrate males showing postprandial hyperglycemia and glycosuria. On the other hand, gonadectomy did alter the character of the alloxan diabetes specifically with respect to fasting oral glucose tolerance and ketonuria.

METHODS

A total of 96 rats of the Wistar strain, equally divided among males and females, was used in the experiment. Prepubertal castration was carried out on 24 animals of each sex at 30 to 45 days of age and postpubertal castration on an equal number at 110 to 125 days of age. This was done to determine whether time of castration might influence reaction to alloxan. Experiments were carried out when the operated animals reached 180 to 220 days of age at which time the males averaged 340 gm. and the females 305 gm. in body weight. That castration had largely eliminated sex difference in body size is indicated by the fact that intact males at this age weigh about 400 gm. and intact females about 240 gm.

Alloxan monohydrate was administered subcutaneously after a 96-hour fast at the rate of 20 mg. per 100 gm. in 3 per cent solution and the animals fed *ad libitum* 15 minutes after injection. Observations were made of blood sugar changes at intervals during the first 61 hours. The severity of the resulting diabetes was evaluated by the estimation of urine sugar and acetone and of postprandial blood sugar 1 and 3 weeks after alloxan and by fasting glucose tolerance at 2 weeks. The analytical methods used were identical with those described in a previous publication. (Beach *et al.*, 1951.)

RESULTS

In these experiments, animals castrated before puberty did not differ from those operated on after puberty. Therefore, in the discussion below,

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the animals are classed together as castrates, no distinction being made as regards time of operation. Among the castrates (Table 1), alloxan produced a fatal reaction or severe diabetes in 75% of the females and only 35% of the males. This greater response of castrate females compared with castrate males, is equal in magnitude and pattern to the sex difference among intact rats (Table 1) previously established in this laboratory (Beach et al., 1951). Chi square techniques were used to determine the statistical significance of the sex differences observed in the castrate and the intact groups. Both showed high significance with P less than .01.

TABLE 1. DIABETES PRODUCED BY ALLOXAN IN CASTRATE AND INTACT RATS
(Number of Animals)

Type Reaction*	Males		Females	
	Castrate	Intact	Castrate	Intact
Fatal	0	12	9	19
Severe Diabetes	17	28	27	45
Mild or Transient Diabetes	9	13	7	2
Resistant	22	19	5	6
Total	48	72	48	72

* Type Reaction.
Fatal—Failed to survive 1 week after injection of alloxan.
Severe Diabetes—Survived at least 1 week with severe hyperglycemia and glycosuria, at 3 weeks postprandial blood sugar was above 270 mg. per cent.
Mild or Transient Diabetes—Showed some symptoms of diabetes but by 3 weeks had recovered or showed postprandial blood sugars below 270 mg. per cent.
Resistant—All others.

The hourly changes in blood sugar during the first 61 hours after alloxan in the castrate rats do not differ from the typical triphasic response observed in intact rats except for the fact that no sex differences in the severity and time of appearance of the hypoglycemic phase were observed.

Table 2 summarizes the data on postprandial blood sugar, urinary glycosuria and occurrence of ketonuria among intact and castrate rats de-

TABLE 2. BLOOD SUGAR, GLYCOSURIA AND KETONURIA IN SEVERELY DIABETIC RATS

Sex and type of animals	Time after alloxan weeks	Postprandial blood sugar mg. per cent	Urine glucose per cent	Animals showing ketonuria, number
Male Castrates	1	411 ±*60(15)†	5.9 ±*1.7(15)†	6(15)†
	3	463 ± 78(11)	5.8 ± 1.4(15)	4(15)
Female Castrates	1	444 ± 63(25)	6.4 ± 1.3(25)	11(25)
	3	531 ±145(16)	6.4 ± 1.4(22)	5(22)
Intact Males	1	402 ± 79(24)	4.7 ± 2.0(26)	3(24)
	3	424 ± 74(23)	5.7 ± 1.1(23)	1(22)
Intact Females	1	426 ± 74(35)	6.3 ± 1.8(39)	20(37)
	3	418 ± 72(28)	7.0 ± 1.8(29)	19(31)

* ± Indicate standard deviation.
† Numbers in parenthesis indicate number of severely diabetic animals upon which the observation was made.

veloping severe alloxan diabetes. Both classes of animals show diabetes of approximately comparable intensity on the basis of postprandial hyperglycemia and glycosuria. It is true that our data show castrate males and females have higher blood sugars three weeks after alloxan than do the corresponding intact controls but the glycosuria was not correspondingly higher and the data are not adequate to either support or disprove the belief that castration influences the severity of diabetic postprandial hyperglycemia. The important contrast between intact and castrate alloxan diabetics is with respect to ketonuria. Intact male diabetics are far less susceptible to ketonuria than are intact female diabetics during the three weeks following alloxan. This sex difference does not appear among rats castrated prior to the development of diabetes. Ketonuria appeared as frequently among diabetic castrate males as among diabetic castrate females and incidence of ketonuria in the castrates at 1 week after alloxan was somewhat less than incidence of ketonuria among intact female diabetics but far greater than among intact male diabetics. Three weeks after alloxan, the castrate diabetics of both sexes showed a tendency to recover from the ketonuria, a tendency not noted among intact female diabetics.

The oral glucose tolerance test (Table 3) given after a 24-hour fast, 2 weeks after alloxan served to reveal another difference in the diabetic state

TABLE 3. FASTING ORAL GLUCOSE TOLERANCE TEST* ON SEVERELY DIABETIC RATS

(Blood Glucose—mg. per cent)

	Males						Females					
	Castrate (15)†			Intact (26)†			Castrate (23)†			Intact (35)†		
Time after glucose (hr.)‡	0	½	2	0	½	2	0	½	2	0	½	2
Highest Value	280	486	362	239	378	265	384	454	480	309	435	329
1st Quartile	255	432	331	138	290	180	250	400	342	215	356	252
Median	189	350	263	94	253	132	211	377	300	123	262	170
3d Quartile	109	284	179	83	224	109	106	306	212	94	235	119
Lowest Value	91	262	136	62	165	62	85	219	101	47	129	44

* The normal tolerance curve at 0, ½ and 2 hr. is 86, 126, 90 mg. % respectively.
† Numbers in parenthesis indicate number of cases.
‡ Rats were fasted 24 hours, after which 100 mg. glucose per 100 gm. body weight were given by mouth.

of intact and castrated rats. It will be noted, in the median, that intact female diabetics have a poorer glucose tolerance than do intact male diabetics, that diabetics of both sexes show a broad spread in tolerance behavior, and some intact diabetics develop severe hypoglycemia when fasted. Castration prior to alloxan yielded diabetics showing an equally broad range of tolerance patterns but there were no instances of severe fasting hypoglycemia. The outstanding effect of castration prior to alloxan was in the elevation of the fasting tolerance curves of both males and females above

those observed in intact animals.[1] The males are outstanding in this regard as seen by comparing the tolerance data of intact and castrated males. In fact, castration so raised the tolerance curves of the male diabetics that they are little different than the tolerance curves of the diabetic castrate females. That castration before alloxan treatment virtually eliminated the sex difference in glucose tolerance of severe diabetics is shown by a comparison of the median and quartile data on male and female castrate diabetics.

DISCUSSION

The failure of gonadectomy to alter the sex difference in susceptibility to alloxan fits into some biological concepts regarding sexuality. Riddle (1931) has summarized evidence of some sex differences not conditioned by the gonads but rather conditioned by the genetic or chromosomal constitution of the individual. In this connection, he points out that castration does not lead to complete reversal of all somatic sex characters because of the persisting influence of the fundamental genic differences of the tissues. Stein (1935–36) showed that castration in 7 day old rats fails to eliminate the sex difference in pituitary gonad stimulating potency observed 17 to 19 days later. Holck and others (1937) found that castration does not entirely eliminate the sex difference of rats in tolerance to certain barbiturates. The persistence of a sex difference in response to alloxan in our experiment suggests one of two possibilities: Either the difference is based upon some fundamental unlikeness in the physiologic constitution of the sexes not dependent on the gonads, or that the gonads predetermine the sex difference prior to 30 to 45 days of age, the time of the earliest castration. It is certain that the difference is maintained for long periods (100 to 150 days) in the absence of gonadal secretion.

That a sex difference exists in the tendency to ketonuria has been amply demonstrated since its first observation by Deuel and Gulick (1932). Castration has been found to eliminate this sex difference in rats (Grunewald,

[1] It has been the practice in this laboratory to give a small dose of insulin to diabetic rats if they appear moribund on the third or fourth day after alloxan. This increases survival by bringing a few animals through the initial crisis. Inasmuch as the present study on castrates followed the more extensive study on intact animals it might be thought that we had become more experienced in postalloxan care and had obtained survival of more severe diabetics which had been lost from earlier studies. This, of course, would tend to increase the number of animals showing high levels of fasting blood sugar and elevated tolerance curves. That this was not the cause of the raised tolerance curves in our castrated groups is indicated by the following: 1. Survival rate was not better at the end than at the beginning of the study on intact rats, showing no evidence of increasing skill in treating the initial crisis.

Castration resulted in an upward shift of the whole range of tolerance curves rather than a mere increase in the number at the top of the range.

Cutler and Deuel, 1934) as regards the metabolism of orally administered diacetic acid and in cats (Chamberlin, Furgason and Hall, 1937) with regard to fasting and dietary ketosis. The present experiments demonstrate that the sex difference in ketonuria in severe alloxan diabetes likewise may be abolished by castration. However, it does not show whether the difference has its basis in a difference in metabolism of ketone bodies or in the kidney threshold of these substances.

We have demonstrated that intact diabetic rats show a marked sex difference as regards fasting blood sugar and corresponding fasting oral glucose tolerance (Table 3). Castration prior to the development of alloxan diabetes was effective in eliminating this sex difference. Furthermore, castration tended in both sexes to yield higher fasting blood sugar among alloxan diabetics than is observed among intact diabetics. It is known that castration in either sex leads to an increase in anterior pituitary size, changes in its histology and content of gonadotropic hormones (Koch, 1937). On the other hand castration has been demonstrated to increase the adrenal weight in male rats and to eliminate sex dimorphism in this regard (Parkes, 1945). Inasmuch as the hormones of pituitary and adrenal cortex play dominant roles in carbohydrate metabolism it seems likely that some rearrangement of the pituitary-adrenal cortical system may be the basis for the observed effects of gonadectomy in these studies.

The broad spread in fasting blood sugar as well as glucose tolerance curves observed in both intact and castrated animals having severe alloxan diabetes has some implications regarding the nature of alloxan damage. Rats treated with alloxan, and exhibiting severe hyperglycemia and glycosuria when fed, show no uniformity of symptoms when fasted. Some quickly develop hypoglycemia while others maintain hyperglycemia under the same conditions. Alloxan diabetic animals present a whole spectrum of different kinds of diabetes and so called "alloxan diabetes" cannot be considered an entity. Abundant evidence has proven that pancreatic islet beta-cells damage occurs consistently when alloxan diabetes is produced and reduction of insulin content of the pancreas, under these conditions, has been shown roughly to correlate with severity of diabetic symptoms (Wrenshall et al., 1950). Failure of insulin production is undoubtedly basic to the production of alloxan diabetes. The present work, however, suggests that the picture in alloxan diabetes may not be this simple for it is impossible to explain the uneven behavior of severe alloxan diabetics on the basis of insulin lack alone. We are inclined to the belief that alloxan may damage, in varying degrees, mechanisms or factors other than insulin which are involved in carbohydrate metabolism. More extensive fundamental studies regarding the nature and breadth of alloxan damage to the organism are needed. It would appear unwise in our present state of knowledge

to interpret, on the basis of insulin lack alone, much of the current work on alterations of metabolism of alloxan diabetic animals and tissues.

SUMMARY

Castration several months before administration of alloxan fails to eliminate the sex difference in incidence of severe diabetes. Castrated females like intact females more frequently develop alloxan diabetes than do castrated or intact males. However, gonadectomy does show some effects upon the character of alloxan diabetes. Male and female castrate alloxan diabetics are about equally subject to ketonuria although there is a marked sex difference in this respect among intact diabetics. Gonadectomy also eliminates the sex difference among alloxan diabetics as regards fasting blood glucose levels and glucose tolerance. Severe alloxan diabetes among castrates is characterized by a poorer fasting glucose tolerance than is alloxan diabetes among intact animals.

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THE PROBLEM OF PLACENTAL PERMEABILITY FOR THYROTROPHIN, PROPYLTHIOURACIL AND THYROXINE IN THE GUINEA PIG[1]

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MANY investigators have reported that the thyroids are enlarged in offspring from mothers treated with antithyroid agents during pregnancy (*mouse*—Kaufman, Hurst and Turner, 1948; *rat*—Hughes, 1944; Goldsmith, Gordon and Charipper, 1945; *guinea pig*—Albrieux, Estefan and Gonzalez, 1946; Webster and Young, 1948; *goat*—Schultze and Turner, 1945; *man*—Davis and Forbes, 1945; Eaton, 1945). This has been taken as evidence for the placental passage of an antithyroid effect.

The placental passage of the antithyroid effect could occur in one or more of three ways (Goldsmith, Gordon and Charipper, 1945). Thyrotrophin (TSH) from the maternal pituitary might cross the placenta to affect the fetal thyroid. D'Angelo and Gordon (1950) have since shown that administration of propylthiouracil is followed by increased concentration of TSH in the serum. Second, the goitrogen might pass the placenta to inhibit thyroid hormone production in the fetus. This would lead to stimulation of the fetal thyroid by TSH secreted by its own pituitary. The third alternative is suggested by the fact that the antithyroid drug decreases production of thyroid hormone by the maternal thyroid. The fetal thyroid might then undergo hyperplasia and supply the hormonal needs of the mother.

The literature concerning the passage of thyrotrophin across the placenta is difficult to interpret. Döderlein (1933), using the guinea pig, and Theresa (1939), using the rat, state that TSH passes the placenta and stimulates the fetal thyroids. Aron (1930), Schittenhelm and Eisler (1935) and Grumbrecht and Loeser (1938), on the other hand, found no consistent evidence for stimulation of the thyroids in guinea pigs born to mothers treated with TSH-active pituitary extracts during pregnancy.

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The permeability of the placenta of the rat for methylthiouracil has been proved by bioassay of the fetuses (Freiesleben and Kjerluf-Jensen, 1947). The passage of other antithyroid agents across the placental barrier has been suggested but not conclusively demonstrated (Hughes, 1944; Goldsmith, Gordon and Charipper, 1945; Albrieux, Estefan and Gonzalez, 1946).

Though it is not concerned with the transmission of the antithyroid effect to the fetus, we felt that passage of thyroxine from mother to fetus should be investigated. There are reports relative to the transfer of thyroid hormone, but they are contradictory. Courrier and Aron (1929) concluded that the placenta of the dog is impermeable to thyroid hormone. Grumbrecht and Loeser (1938) and Theresa (1939), on the other hand, state that this hormone passes the placenta in the guinea pig, rat, and rabbit.

In a single study we have attempted to clarify the problem of the permeability of the guinea pig placenta for thyrotrophin, propylthiouracil and thyroxine.

METHODS

Working under the assumption that the placental passage of the antithyroid effect is due to one or more of three possibilities, the following series of experiments was performed.

Thyroidectomy of the mother removes the endogenous supply of thyroid hormone. It is presumed from the work of D'Angelo and Gordon (1950) on the female guinea pig that production of TSH by the maternal pituitary would thus be released from inhibition and elevate the level of circulating TSH. The level of TSH can be further raised by injection of this hormone. If TSH were to pass the placenta of a pregnant animal so treated, the thyroids from the offspring should show some change. To test this hypothesis, 8 female guinea pigs were thyroidectomized at least 30 days prior to mating. Beginning the 38th day after copulation they were given daily subcutaneous injections of 0.5 mg. of purified thyrotrophic hormone from a preparation containing 10 Junkmann-Schoeller units per mg. If there were no changes in the fetal thyroid, the placental passage of an antithyroid effect could not be due to the transfer of TSH from mother to fetus. Furthermore, the possibility that it had undergone hyperplasia and compensated for the deficiency of thyroid hormone in the mother would be eliminated.

It is known that the thyroids of offspring from propylthiouracil-treated guinea pigs are hyperplastic (Webster and Young, 1948). The hyperplasia could be shown to be due to the placental passage of propylthiouracil, provided there were no hyperplasia of the fetal thyroids following treatment of thyroidectomized mothers with TSH. In order to make this comparison, 11 pregnant guinea pigs were given approximately 25 mg. propylthiouracil per day in the drinking water from the 38th day of pregnancy until parturition.

The problem of the passage of thyroxine from mother to fetus was investigated in another series of experiments. Dempsey and Astwood (1943) demonstrated that the effects of thiouracil on the thyroid of the adult rat can be counteracted by small daily doses of thyroxine. If it were shown that propylthiouracil acts directly on the fetus, an amelioration of the antithyroid effect by thyroxine administered to the mother during

gestation would be convincing evidence that thyroxine can enter the fetal circulation from the mother. To test this hypothesis, 4 pregnant animals were given 25 mg. of propylthiouracil per day in the drinking water and injected daily with 0.025 mg. thyroxine[3] in saline beginning the 38th day of pregnancy.

The placental passage of thyroxine might be shown in another way. The administration of thyroid hormone to an intact adult animal causes flattening of the thyroid epithelium and enlargement of the follicles (Courrier, 1928; Adams and Jensen, 1944). If thyroxine passes the placental barrier, the administration of this substance to pregnant guinea pigs might cause similar changes in the fetal thyroids. This possibility was tested in two groups of 5 females each. Both were treated with different doses of thyroxine for at least 30 days before mating and throughout pregnancy. One group received subcutaneous injections of 0.10 mg. thyroxine every 4 days. The other was injected with 0.15 mg. thyroxine every 3½ days.

The offspring from 13 untreated pregnant guinea pigs served as controls for all of the above experiments.

On the day of delivery the thyroids and pituitaries from 71 offspring from treated and 38 offspring from untreated mothers were removed, dissected free of excess fat, weighed on a torsion balance, and fixed in Dawson's fixative (Dawson and Friedgood, 1938). The thyroids were dehydrated and embedded by the dioxan-paraffin method, sectioned at 7 micra, stained with hematoxylin and eosin, and examined microscopically.

The guinea pigs used in this study were from our own stock kept under the following conditions: The temperature of the laboratory was maintained between 70° and 75° F. Food and water were available at all times. The diet consisted of a mixture of oats and rabbit pellets supplemented with alfalfa hay and fresh cabbage once daily.

RESULTS

Three of the 8 thyroidectomized-TSH-injected females delivered living litters. On gross examination at autopsy no regenerated thyroid tissue was found in these mothers. The thyroids of their offspring showed neither an increase in weight (Table 1), nor any histological evidence of TSH stimulation (compare Figs. 1 & 2). Thus, there is no evidence that the antithyroid effect in the fetus is due to passage of TSH from the mother or to hyperplasia of the fetal thyroid which might have supplied thyroid hormone to the mother.

As in other work (Webster and Young, 1948), administration of propylthiouracil to the mother during pregnancy caused extreme hypertrophy and hyperplasia of the fetal thyroid (Table 1 & Fig. 3). Since no such effect was found in offspring from thyroidectomized females treated with TSH, the thyroid enlargement in offspring from propylthiouracil-treated mothers must have been due to placental passage of the antithyroid drug. The stimulation of the fetal thyroid by thyrotropin from its own pituitary is borne out by the fact that the fetal pituitaries showed a simultaneous increase in weight (Table 1).

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[3] In this and the following experiments either synthetic d-l thyroxine (Roche-Organon) or thyroxin crystals (Squibb) was used.

TABLE 1. EFFECT OF TREATMENT OF THE MOTHER ON THE WEIGHT OF THE THYROID AND PITUITARY OF THE NEWBORN GUINEA PIG

Treatment	No. mothers	No. young	Birth weight	Thyroid weight (mg./100 gm.)	Pituitary weight (mg./100 gm.)
Untreated controls	13	38	93.8	28.7 ± 1.0[1]	5.2 ± 0.1[1]
Thyroidectomized plus 5 J-S units TSH daily for 30 days	3	5	112.4	27.1 ± 1.1	5.0 ± 0.6
Propylthiouracil, 25 mg. daily for 30 days	11	20	94.9	2090.3 ± 301.3*	8.4 ± 0.4*
Propylthiouracil, 25 mg. and 0.025 mg. thyroxine daily for 30 days	4	14	97.7	267.9 ± 50.8*	5.7 ± 0.3
Thyroxine, 0.10 mg. every 4 days throughout pregnancy	5	20	94.8	21.5 ± 0.8*	4.8 ± 0.2
Thyroxine, 0.15 mg. every 3½ days throughout pregnancy	5	12	101.1	17.4 ± 1.1*	4.3 ± 0.3*

[1] The ± values are the standard errors of the means.
* The difference from the control value is significant at the 1% level of confidence ("students" t).

When propylthiouracil and thyroxine were administered simultaneously during pregnancy, the fetal thyroids were larger than normal, but significantly smaller than in the group receiving the antithyroid drug alone (Table 1). Histologically they showed less drastic effects. Although the epithelium was high, colloid-containing follicles were formed (compare Figs. 3 & 4). The pituitaries were of normal weight. All of this indicates a partial inhibition of the production of TSH by the fetal pituitary in consequence of the passage of the injected thyroxine across the placenta. Theoretically, if sufficient thyroxine were administered with the propylthiouracil the fetal thyroids should be normal.

Further evidence for the passage of thyroxine across the placenta is considered to be given by data from the offspring of thyroxine-treated mothers. The thyroid epithelium was not flattened nor was there enlargement of the follicles as we might have expected from work of Courrier (1928) and Adams and Jensen (1944), but the thyroids were significantly smaller than those of control newborn (Table 1). The greater the dose of thyroxine, the smaller were the thyroids. The pituitaries showed corresponding changes in weight. Histologically, the thyroids of offspring from thyroxine-treated mothers were similar to the controls except for the presence of smaller follicles and relatively more interfollicular cells and connective tissue (compare Figs. 1, 5, 6). Since the pituitaries and thyroids of these offspring were significantly smaller than those of the control newborn, it is assumed that the TSH production by the fetal pituitary exerts an influence on the normal prenatal development of the thyroid.

DISCUSSION

The data indicate that the enlargement of the thyroids in offspring from

propylthiouracil-treated female guinea pigs is due neither to passage of TSH from the mother to the fetus nor to hyperplasia of the fetal thyroid which might compensate for the deficiency of thyroid hormone in the mother. It has been concluded, therefore, that of the three hypotheses

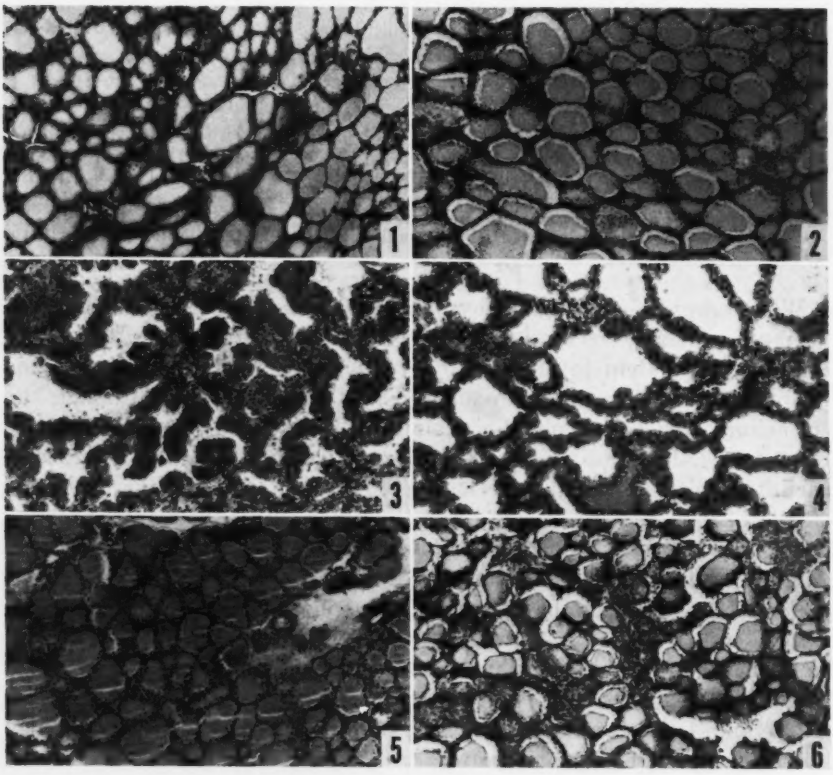

PLATE 1

FIG. 1. Thyroid of an offspring from an untreated control female. ×150.

FIG. 2. Thyroid of an offspring from a thyroidectomized female injected with 5 J-S units TSH daily the last 30 days of gestation. ×150.

FIG. 3. Thyroid of an offspring from a female treated with 25 mg. propylthiouracil daily the last 30 days of gestation. ×150.

FIG. 4. Thyroid of an offspring from a female treated with 25 mg. propylthouracil and 0.025 mg. thyroxine daily the last 30 days of gestation. ×150.

FIG. 5. Thyroid of an offspring from a female injected with 0.10 mg. thyroxine every 4 days throughout gestation. ×150.

FIG. 6. Thyroid of an offspring from a female injected with 0.15 mg. thyroxine every 3½ days throughout gestation. ×150.

which might have accounted for the transmission of the antithyroid effect to the fetus, the placental passage of propylthiouracil is most in accord with the data.

This conclusion is consistent with the opinions of several investigators who have studied one or more aspects of the subject (Freiesleben and Kjerluf-Jensen, 1947, for the goitrogen methylthiouracil in the rat; Aron, 1930, Schittenhelm and Eisler, 1935, Grumbrecht and Loeser, 1938, for TSH in the guinea pig; Grumbrecht and Loeser, 1938, Theresa, 1939, for thyroid hormone in the guinea pig and rabbit).

An explanation for the contradictory reports of the placental passage of thyrotrophin and thyroxine is not easy. Döderlein (1933) stated that TSH passes the placenta to cause hypertrophy of the fetal thyroid in the guinea pig. In our opinion, his data do not support this conclusion. From a comparison of his figures of alleged TSH stimulation with his control figure, it might seem that the conclusion was justified. Close examination of the thyroid of the newborn control he chose for comparison (his Fig. 2a) reveals, however, that the epithelium is squamous and that the follicles are distended. This is a condition we have not seen in any one of a large number of normal newborn guinea pigs. Moreover, the appearance of the fetal thyroids which he believed to have been subjected to TSH stimulation was not different from that in our controls. Species differences may account for the reported passage of TSH through the placenta of the rat (Theresa, 1939), and for the non-passage of thyroid hormone across the placenta of the dog (Courrier and Aron, 1929).

Since it is clear that thyroid hormone and at least two antithyroid agents pass the placenta, the long term effects on the young from females treated with these substances during pregnancy become of interest. Rather severe chronic hyperthyroidization of the pregnant rat has been reported to produce cretinoid progeny (Cunningham, 1941). The retarded growth of the cretinoid rats was overcome by the administration of thyroxine. We have seen no such condition in the guinea pig. The weight of newborn animals from mothers given thyroxine during pregnancy was not less than that of the controls (Table 1). Following another study of thyroxine-treated pregnant guinea pigs (Peterson, Rayner, Brown and Young, 1950), the development of 9 male and 18 female offspring was observed for 3 to 4 months after birth. Their growth and sexual maturation were not different from the controls.

Changes in the goiters in the offspring of propylthiouracil-treated female guinea pigs have been followed (Webster, 1949). The thyroids underwent a rapid involution, but were still enlarged even after $8\frac{1}{2}$ months. The presence of these goiters had no apparent effect on growth and sexual maturation. Thus, even though the weight of the thyroids and

pituitaries in offspring from animals treated similarly differed significantly from normal (Table 1), there was no other effect on postnatal development.

Antithyroid agents have been used in the treatment of hyperthyroidism complicating pregnancy in the human female. Varied effects on the fetus and newborn have been reported. Davis and Forbes (1945) found an enlarged thyroid gland in a six-month fetus from a mother treated with thiouracil. An infant observed by Eaton (1945) had a grossly enlarged thyroid which regressed in size until it was no longer visible by the third month. Whitelaw (1947) found little difference from normal in the thyroid of a male monster born to a mother who had received thiouracil during the latter part of pregnancy. Caren (1949) states that there were no ill effects of thiouracil on the fetus or its subsequent development. We suggest from our work on the guinea pig that the thyroid enlargement in some infants born to mothers treated with antithyroid drugs can be attributed to placental passage of the drug.

SUMMARY AND CONCLUSIONS

The thyroids and pituitaries of offspring from thyroidectomized female guinea pigs treated with thyrotrophin during pregnancy did not differ in weight from those of controls, nor was there any histological sign of thyrotrophic stimulation of the fetal thyroid. Administration of propylthiouracil during pregnancy causes hyperplasia of both the fetal thyroid and pituitary which can be counteracted by simultaneous administration of thyroxine. Both the fetal thyroid and pituitary are suppressed in development when thyroxine alone is injected into pregnant animals. Such results indicate that the placenta of the guinea pig is impermeable to thyrotrophin, but is permeable to both propylthiouracil and thyroxine.

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INACTIVATION OF ADRENOCORTICOTROPIC HORMONE IN VITRO BY TISSUES*

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THE fact that frequent injections of adrenocorticotropic hormone (ACTH) are necessary in order to achieve a maximum physiological or clinical effect, along with the rapid disappearance of adrenocorticotropic activity from plasma which has been demonstrated to occur after intravenous injection of the hormone (Sayers *et al.*, 1949; Greenspan *et al.*, 1950), and the inability of Richards and Sayers (1951) to find hormonal activity in the urine, indicate that the hormone is rapidly destroyed or utilized in the organism. In order to determine the contributions of various tissues to the loss of ACTH activity, a study was made of the inactivation of the adrenocorticotropic hormone in vitro by organ slices and homogenates.

METHODS

The data reported here were obtained in two separate series of experiments.** The first series was conducted in 1947–1948, and some of the results of these experiments have been reported elsewhere in a preliminary note (Li, 1948).

The adrenocorticotropic hormone used in all studies was isolated from sheep pituitaries by the method of Li, Simpson and Evans (1943). Two ACTH peptide preparations were used: Preparation L 2119 M is the 5% trichloroacetic acid (TCA) soluble fraction obtained after pepsin digestion (Li and Pedersen, 1950); preparation L 2145 MP, the 5%–25% TCA insoluble peptide fraction obtained after such hydrolysis (Li, 1951).

In the first series of experiments, the adrenal ascorbic acid depletion method of Sayers, Sayers and Woodbury (1948) as modified by Munson *et al.* (1948) was employed for the assay of ACTH potency. The hormone was administered intraperitoneally instead of intravenously, and the adrenal ascorbic acid concentrations were determined by the dye method (Bessey, 1944). In the latter series, the original procedure of Sayers *et al.* (1948) was employed, with a modification of the method of Roe and Kuether (1943) used for the adrenal ascorbic acid determination (Geschwind *et al.*, 1951).

Tissues were obtained from 40 day old male rats of the Long-Evans strain. Homogenates were prepared by the technique of Potter and Elvehjem (1936), and slices by the Martin (1942) slicer. The particulate fractions were isolated in 0.25 M sucrose by the method of Schneider and Hogeboom (1950). The reaction mixtures were shaken con-

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stantly in a constant temperature bath. In the first series of experiments, 2 cc of 1% homogenates in pH 7.4 Krebs' solution were incubated with 2 mg hormone at 37.4° C for 60 minutes and the mixtures were then diluted to a desired concentration for assay. When slices were employed, 20 mg of the tissue in 2 cc buffer were used. Finally, 2 mg of the hormone were incubated with 2 cc plasma under the same conditions. Later experiments employed 2.5 mg of the hormone which were incubated in 5 cc of pH 7.4 Krebs-Ringer-phosphate buffer with 100 mg of tissue slices or hemidiaphragm, for 30 minutes at 34.0° C. The same amount of hormone was incubated for 1 hour in 5 cc of 3–4% homogenate prepared with Krebs-Ringer-phosphate solution.

Particulate fractions were made up in the same proportions as they exist in tissue, with the supernatant fraction adjusted to contain 1 mg particulate nitrogen in 4 cc of pH 7.4 Krebs-Ringer-phosphate solution containing 0.25 M sucrose. Incubation was for 1 hour at 37.4° C with 2.5 mg of the hormone preparations.

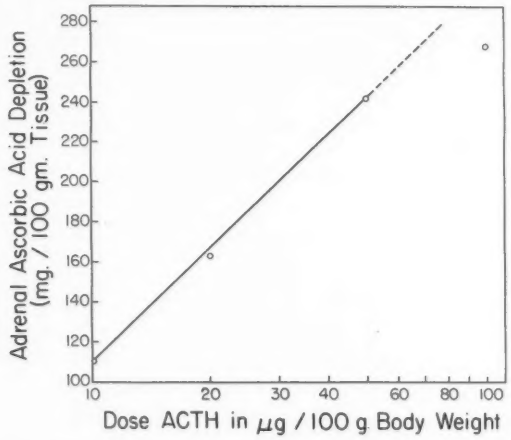

FIG. 1. Depletion of adrenal ascorbic acid following intraperitoneal administration of ACTH in hypophysectomized male rats.

RESULTS

In order to effect a semi-quantitative analysis of the results obtained in the first series of experiments, it was necessary to determine a dose-response relationship when the hormone was injected intraperitoneally. It can be seen in Figure 1 that a straight line relationship obtains between the adrenal ascorbic acid depletions and the logarithm of the injected dosages (0.01–0.05 mg.) of ACTH protein. It is of interest that the magnitude of depletions obtained by intraperitoneal injection at higher dosages (Table 1) far exceeds those obtained by comparable doses (0.05–0.10 mg.) using the intravenous route of administration of the hormone.

The data obtained in the first series demonstrate that incubation of the hormone with liver slices and with liver, kidney and adrenal homogenates

TABLE 1. STANDARDIZATION OF SHEEP ADRENOCORTICOTROPIC HORMONE BY THE ASCORBIC ACID METHOD

(Intraperitoneal Injection)

Dose	No. of rats	Ascorbic acid concentration	Depletion
mg.		mg./100 mg. adrenal	mg./100 g. adrenal
0.00	51	0.476 (0.430–0.556)*	
0.01	14	0.366 (0.297–0.410)	110
0.02	21	0.312 (0.299–0.339)	164
0.05	9	0.235 (0.224–0.296)	241
0.10	4	0.208 (0.152–0.269)	268

* Values in parentheses represent the range of level obtained.

resulted in a marked loss of activity (Table 2). It may be noted that though inactivation of the hormone is effected by both liver slices and homogenates, the latter appear more effective. Comparatively little inactivation was found when the hormone was incubated with rat plasma.

TABLE 2. THE INACTIVATION OF ACTH BY VARIOUS TISSUES

(1st Series)

Experiment	Dose	No. of rats	Average ascorbic acid depletion	ACTH activity remaining
	mg.		mg./100 gm. adrenal	%
Liver Slices	0.02	8	75	35
	0.10	8	180	25
Liver Homogenates	0.02	7	20	<50
	0.10	8	46	<10
Heated Liver Homogenate*	0.02	4	158	93
	0.10	4	186	25
Kidney Homogenate	0.02	4	+25†	<50
	0.10	4	2	<10
Adrenal Homogenate	0.02	4	0	<50
	0.10	8	54	<10
	0.20	4	52	< 5
Plasma	0.02	8	134	70

* The homogenate was placed into a boiling water bath for 15 minutes before incubation.
† A+value denotes an ascorbic acid concentration in the experimental adrenals greater than that observed in controls.

It is of interest to note that the heating of liver homogenates does not abolish completely the ability of liver to inactivate ACTH. A further investigation into the residual potency for inactivation was therefore carried out in the later experiments.

The results of the second series of experiments amply confirmed the

earlier findings.[1] Thus, both liver and kidney slices inactivated the hormonal potency of ACTH, with kidney producing the greater inactivation (Table 3). Furthermore, diaphragm has also been shown to inactivate ACTH, though it also is less effective than the kidney. Liver homogenates or diaphragms after heat-treatment show only partially destroyed effectiveness in inactivating the hormone.

TABLE 3. THE INACTIVATION OF ACTH BY LIVER, KIDNEY AND DIAPHRAGM
(2nd Series)

4 rats in each experiment

Experiment	Dose	ACTH activity remaining
	mg.	%
Liver Slices	0.0065	6
Kidney Slices	0.0065	<3
Diphragm	0.0065	23
Heated Diaphragm*	0.004	54
Liver Homogenate	0.003	<7
Heated Liver Homogenate*	0.003	23

* The diaphragm or homogenate was placed into a boiling water bath for 15 minutes before incubation.

In order to ascertain the cause for the residual inactivation potency of heated tissues, the following experiments were carried out. Heated diaphragms were incubated with 2.5 mg ACTH for 60 minutes at 37.4° C. The medium was assayed and only 30% of the original activity was found to remain. The diaphragm residues were then washed repeatedly with ice-cold saline solution. The washed residues were homogenized with either 5 cc of 0.1 N HCl or 0.1 N NaOH, and the homogenate filtered. The filtrates were then neutralized and assayed, and showed approximately 15% of the original activity. Thus, it appears that a portion of the activity was "inactivated" by adsorption or binding, and that such activity could be recovered by elution with strong alkali or acid. If such inactivation does occur, it should be demonstrable in a short period of incubation, and also at lower temperatures. Liver homogenates were therefore incubated with 2.5 mg protein at 25° for only 5 minutes; assay of the medium showed less than 1 per cent of the original activity remaining. The effect of temperature was demonstrated by incubating 2.5 mg protein with 100 mg liver slices in 5 cc medium for 60 minutes, with 1 group incubated

[1] It should be re-emphasized that the assay procedure employed in the second series of experiments is the original method of Sayers *et al.* (1948). In the case of the earlier experiments, reported in Table 1 and Figure 1, the minimal effective dose of hormone was considered to be 0.01 mg.; in contrast, the assay procedure employed in this later series permits the determination of 0.0002 mg. of a standard ACTH preparation.

at 38.1° C and another 6° C (cold-room). The results of 3 individual experiments demonstrated that less than 6% of the original activity remained after incubation at the higher temperature, while only 25% was found at the lower temperature. It appears reasonable to assume, therefore, that adsorption or binding by the tissue is responsible in part for ACTH inactivation.

On the other hand, it is beyond doubt that inactivation is to a large measure produced by a heat-sensitive system. It was, therefore, of interest to investigate which particulate fractions of liver homogenates are responsible for the inactivation.

Experiments using various particulate fractions obtained by differential centrifugation are summarized in Table 4. Nuclei and microsomes appear to possess little activity in the inactivation of ACTH. Mitochondria have consistently shown greater inactivation potency than either the nuclei or

TABLE 4. INACTIVATION OF ACTH BY LIVER PARTICULATES

Fraction	Dose	No. of rats	ACTH activity remaining
	mg.		%
Nuclei	0.003	6	36
Mitochondria	0.050	21	17
Microsomes	0.005	12	46
Supernatant	0.050	16	3
Heated Supernatant*	0.005	8	39
Microsomes and Supernatant	0.050	7	2

* The supernatant was placed into a boiling water bath for 15 minutes before incubation.

microsomes, but the greatest activity, by far, resided in the supernatant fraction. It should be noted that the percentage of inactivation obtained with a heated supernatant fraction is comparable to that obtained with nuclei and microsomes. However, the total homogenates, from which the fractions were prepared, almost completely inactivated the hormone. No

TABLE 5. COMPARATIVE INACTIVATION OF ACTH PROTEIN AND PEPTIDE PREPARATIONS*

Preparation	Inactivating agent	No. of rats	ACTH activity remaining‡
			%
Protein† L2117C	Liver Homogenate	9	2
Peptide† L2145MP	Liver Homogenate	9	<0.4
Protein L2071ABP	Supernatant fraction	6	18
Peptide L2119M	Supernatant fraction	6	5

* The homogenate and supernatant were incubated for 60 and 30 minutes, respectively with 2.5 mg. protein or peptide at 37.4° C.

† Average of two separate experiments.

‡ The hormone was assayed in all instances at the dosage of 0.05 mg. per 100 gm. body weight.

single fraction, nor a combination of the supernatant and microsome fractions, was capable of duplicating these results.

Finally, it was deemed important to determine whether any quantitative difference existed between protein and peptide ACTH preparations with respect to their inactivation by tissues. For this purpose, four different protein and peptide preparations were employed. The results, summarized in Table 5, seem to indicate a more rapid inactivation of peptide fractions than of protein.

DISCUSSION

With the probable exception of plasma, all the tissues studied were capable of causing inactivation of ACTH. This is not surprising in the light of the known widespread distribution of cathepsins and other proteolytic enzymes. It would, therefore, be premature to presume the presence of any specific—for the inactivation of ACTH—enzyme in any tissue, such as has been suggested in liver for insulin: the "insulinase" of Mirsky and Broh-Kahn (1949). Similarly, there would appear to be no reason for ascribing any special importance to the ability of the adrenal homogenate to inactivate the tropic hormone for part of that organ. Inactivation of a tropic hormone by its target organ has been previously described in gonadotropic hormone inactivation by ovarian tissue (Seidlin, 1940), and in the destruction of thyrotropic hormone activity by the thyroid (Seidlin, 1940; Rawson, Sterne and Aub, 1942).

The marked inactivation by kidney is of interest with regard to the results of Richards and Sayers (1951), who found a considerable amount of activity localized in the kidney shortly after the injection of ACTH, although all attempts to demonstrate activity in urine, after ACTH administration, were unsuccessful.

It should be noted that liver homogenate is more effective in inactivating ACTH than are liver slices. Analogous effects have been obtained by Sayers (1951), who observed that ACTH activity was not destroyed at 37° C by anterior pituitary tissue as long as the integrity of the cells was maintained. When the cells of this tissue were crushed, the ACTH activity which had been incubated with it disappeared. Such results with homogenates would anticipate the successful use of tissue particulate fractions, either alone or in combination, for the inactivation of ACTH.

A heat-resistant system capable of causing ACTH inactivation has been demonstrated in tissues, and it has been postulated that such inactivation involves adsorption or binding of activity by tissues. Binding of a hormone by tissue has been investigated by Stadie *et al.* (1949), who clearly demonstrated the interaction of tissues with insulin.

The presence of peptidases and proteases in anterior pituitary prepara-

tions has been demonstrated by Adams and Smith (1951), and it is conceivable that those enzymes which may accompany the ACTH are the source of the residual inactivation potency still found after heating tissues, since the hormone was not heated along with the tissue. However, the apparent failure of plasma to inactivate the hormone, and the fact that all our ACTH preparations are boiled for 2–3 hours at pH 7.5 during their initial purification (Li *et al.*, 1943), argue against such a possibility.

On the other hand, the aforementioned heating of our ACTH preparations, which can be expected to destroy all accompanying enzymatic activity, may help explain the discrepancies between one of our results and others found in the literature. We have found little inactivation of ACTH by rat plasma, which is a finding consistent with that of Richards and Sayers (1951) to the effect that rat ACTH was stable in vitro in heparinized rat blood for one hour at 37° C. These results do not conform with the conclusions of Reiss and co-workers (1951) that ACTH is 75% inactivated in heparinized 80% rat plasma in 5 minutes at 37° C, or with the results of Pincus (1951), demonstrating the disappearance of a large proportion of activity when ACTH is incubated with whole blood. Our preparations are boiled as outlined above, and Richards and Sayers have boiled their preparations in 0.1 M HCl before incubation. It is noteworthy that Sayers (1951) states that before heating in acid was employed by him, his "studies indicated that ACTH was rather unstable in blood." Whether Reiss *et al.* (1951) or Pincus (1951), boiled their preparations is not known; neither is their method of preparation of ACTH, nor the species from which their ACTH was derived, nor the concentration at which the ACTH was incubated with plasma or blood. Any or all of these factors may be the cause of the differences reported.

SUMMARY

Incubation of ACTH protein or peptide preparations with liver and kidney slices, diaphragm, and homogenates of liver, kidney and adrenal results in inactivation of the hormone, with the rate of inactivation of peptide preparations probably greater than that of proteins. Boiled tissues only moderately inactivated the hormone. The supernatant fraction of liver, obtained by differential centrifugation, possessed most of the inactivating potency of this organ. It has been hypothesized that the ability of tissue to inactivate ACTH, even after heating, can be explained by the binding or adsorption of the hormone to the particular tissue.

Incubation of ACTH with rat plasma did not result in a loss of activity. The discrepancy between our results and those of others in this regard, is discussed.

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THE EFFECT OF A SINGLE INJECTION OF AN ANTITHYROID DRUG ON HYPERPLASIA IN THE THYROID OF THE CHICK EMBRYO[1]

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THE discovery of the powerful goitrogenic effects in rats following the administration of sulfonamides, thiocarbamides and thiouracils has led to the investigation of the reactions to these substances in many different animals and in man (MacKenzie, MacKenzie and McCollum, 1941; Richter and Clisby, 1941; Kennedy, 1942; Astwood, Sullivan, Bissell and Tyslowitz, 1943; MacKenzie and MacKenzie, 1943). The characteristic morphological changes produced in the thyroid gland by these drugs are a loss of colloid, hyperemia, and an enlargement (hypertrophy) and multiplication of cells (hyperplasia) leading to the increased size and weight of the gland. The exact mode of action of antithyroid compounds is not known but evidence supports the theory that the thiocarbamides among them prevent the iodination of tyrosine, the second stage in the synthesis of the thyroid hormone (Pitt-Rivers, 1950). The hypertrophy and hyperplasia of the thyroid following treatment with these drugs are evidently responses to the excessive secretion of thyrotrophic hormone by the anterior pituitary gland that results from the decreased amount of thyroid hormone in the circulating blood.

Although the activity of these thyroid inhibiting compounds has been most widely studied in the rat, considerable research has been devoted to their effects on the chick. The first tests of the action of some sulfonamides gave negative results (MacKenzie and MacKenzie, 1943). However, later it was reported that enlargement of the thyroids of one to two day old chicks, occurred after treatment with 0.1% thiourea and thiouracil for 14 days (Mixner, Reineke and Turner, 1944; Schultze and Turner, 1945). Tests of several thiourylenes in chicks produced an even more marked response than in mammals (Astwood, Bissell and Hughes, 1944). After 10 weeks of treatment, the most active of these compounds caused thyroid weights averaging 45 times those of the controls. Other experi-

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ments have been reported with substantially the same results (see review of Charipper and Gordon, 1947).

The work of Grossowicz (1946) emphasized delayed hatching and yolk sac retraction in chick embryos following injections of thiourea. Further study of the effects of antithyroid drugs in chick embryos was made by Adams and Bull (1949). They injected thiourea and thiouracil (Deracil) into the yolk sacs of developing chicks. The results were retardation of hatching, lack of retraction of the yolk sac, decreased body and limb growth, enlargement of the thyroid gland with typical hyperemia, hypertrophy and hyperplasia, a marked increase in height of follicular epithelium, vacuolization of the colloid and colloid depletion. McCreight (1950) reported similar reactions in another strain of chicks. Even in chicks hatched from eggs of hens fed with thiouracil the thyroid glands were heavier than those of control chicks (Andrews and Schnetzler, 1945; McCartney and Shaffner, 1949).

Although hypertrophy and hyperplasia of the thyroid have been reported to result from treatment with antithyroid agents in a variety of fishes, amphibians, reptiles, birds and mammals, the majority of these experiments have emphasized the increased size of the follicular cells and weight of the thyroid, i.e., *hypertrophy* (often termed hyperplasia) rather than increase in cell numbers, i.e., *hyperplasia*, on which the increased size of the gland largely depends (review by Charipper and Gordon, 1947; also, Adams and Bull, 1949; Adams and Craig, 1949, 1950, 1951; D'Angelo, Gordon and Charipper, 1947; Domm and Blivaiss, 1948; Goldsmith, 1949; Joel, D'Angelo and Charipper, 1949; Lever, Miltenburg and van Oordt, 1949; Lynn and Wachowski, 1951; McCreight, 1950; Ratzersdorfer, Gordon and Charipper, 1949). However, a few observers have recorded conspicuous mitotic activity shortly after the administration of goitrogenic compounds (Paschkis, Cantarow, Rakoff and Rothenberg, 1945; Adams and Bull, 1949). Paschkis *et al.* (1945) found that thiouracil given daily to rats in the drinking water produced a hyperplasia (cell multiplication) as soon as 24 hours after treatment began. The hyperplasia rose to a maximum at 10 days and began to fall at 15 days.

The present investigation was undertaken to determine the amount of mitotic activity in chick embryos given one injection of an antithyroid drug on the 14th day of incubation and killed at 24 hour intervals thereafter, compared with that in controls killed at similar intervals. The 14th day was chosen for the single injection because on the 13th day the follicles of the thyroid have become distinct and contain colloid (Bradway, 1929) and by the 15th day the gland has attained essentially the condition that it maintains until hatching (Sun, 1932; Martindale, 1941; Bull, 1948).

METHODS

On the 14th day of incubation, White Plymouth Rock chick embryos were injected with a dose of 0.5 cc. of a 0.4% solution of thiourea[2] or a 0.2% solution of 6-methyl thiouracil[2] or propyl thiouracil;[2] or, as controls, with 0.5 cc. of distilled water or left untreated. The solutions were injected into the yolk sac (Adams and Bull, 1949). At intervals of 24 hours through the 21st day of incubation, three to six embryos from each group were weighed and their thyroids removed, weighed, fixed in Bouin's fluid, sectioned at 5μ and stained in Mallory's triple connective tissue stain. From the 93 embryos, there were 83 thyroids that could be used for histological study and determination of mitotic activity (table 1).[3] Mitoses were counted in an area of 0.02 sq. mm. in 10 sections (a total area of 0.2 sq. mm.) selected from the middle of the gland. Each of the sections counted was separated by at least five sections from any other on which a count was made.

RESULTS AND DISCUSSION

Although the main interest of this investigation concerned the mitotic activity in the thyroid glands of chick embryos given a dose of an antithyroid drug, certain other observations deserve comment.

Effect of antithyroid drugs on hatching and yolk sac retraction: Since the experiment ended at the same time as the usual incubation period, little could be learned about delay in hatching or retraction of the yolk sac. However, no chicks injected with thiourea or propyl thiouracil hatched on or before the 21st day of incubation and the two opened on the 22nd day after treatment with thiourea (not included in table 1) gave no sign of hatching. Moreover, among the chicks injected with these two drugs, only one of those treated with thiourea had the yolk sac partially retracted. This chick was one of the two dissected on the 22nd day of incubation. In contrast to this, of the nine chicks dosed with methyl thiouracil and examined on the 20th and 21st days of incubation, four had started to break through their shells,—two on the 20th day and two others on the 21st day when the controls were hatching; in two, the yolk sacs were entirely within the body.

The results with thiourea and propyl thiouracil generally confirm those of Grossowicz (1946), Adams and Bull (1949), McCartney and Shaffner (1949) and McCreight (1950). These two drugs delay hatching and retrac-

[2] Thiourea (thiocarbamide, $CS(NH_2)_2$) was purchased from Eimer and Amend, New York City, and 6-methyl thiouracil and propyl thiouracil from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] In addition a series of chicks was treated as above but some hours prior to dissection 0.5 cc. of a 0.1% or 0.01% solution of colchicine was injected into their yolk sacs. This drug proved toxic in many cases so that of the 55 chick embryos in the series, only 31 were available for study. Although the observations on these chicks are not included, the data on body weights, thyroid weights and mitotic counts in general were similar to those for the chicks discussed in this paper, but the mitoses being blocked by colchicine were much more abundant.

TABLE 1. DATA CONCERNING CHICK EMBRYOS GIVEN ONE DOSE OF 0.5 CC. OF 0.4% THIOUREA OR 0.2% METHYL THIOURACIL OR 0.2% PROPYL THIOURACIL BY INJECTION INTO THE YOLK SAC ON THE 14TH DAY OF INCUBATION AND KILLED AT INTERVALS OF 24 HOURS THEREAFTER. CONTROLS RECEIVED 0.5 CC. OF DISTILLED WATER OR WERE UNTREATED.

(% = percentage difference compared with controls.)

	Controls					Thiourea-injected				
Day killed	No. embryos	Av. body wt. gm.	Av. thy. wt. mg./100 gm. body wt.	No. mitoses/0.2 sq. mm. thyroid	No. mitoses/thy. wt. in mg./100 gm. body wt.	No. embryos	Av. body wt. gm.	Av. thy. wt. mg./100 gm. body wt.	No. mitoses/0.2 sq. mm. thyroid	No. mitoses/thy. wt. in mg./100 gm. body wt.
15	2	11.5	8.6	25.5	229,950	3	15.7 +36.5%	6.0 −30.2%	16.7 −34.5%	103.634 −54.9%
16	3	18.7	8.7	28.0	242,467	4	17.6 −5.9%	12.1 +40.0%	38.3 +36.8%	457,150 +88,5%
17	4	17.6	11.5	20.1	230,575	3	20.8 +18.2%	19.9 +73.0%	36.0 +79.1%	709,000 +207.5%
18	2	25.8	10.1	16.5	177,900	4	22.0 −14.7%	20.5 +103.0%	28.3 +71.5%	618,600 +247.7%
19	3	26.7	12.0	14.0	179,600	2	23.8 −10.9%	29.4 +145.0%	16.5 +17.9%	536,250 +198.6%
20	2	35.5	11.8	20.5	240,650	4	23.4 −34.1%	33.4 +183.0%	15.8 +22.9%	649,575 +169.9%
21	2	29.0	13.0	17.0	218,200	3	25.3 −12.8%	32.3 +148.5%	16.3 −4.1%	585,800 +168.5%
	Methyl thiouracil-injected					Propyl thiouracil-injected				
15	1	16.5 +34.8%	7.5 −20.0%	35.0 +37.3%	262,500 +14.2%	3	14.7 +27.8%	7.5 −12.8%	3.0 +29.4%	253,134 +10.1%
16	4	17.3 −7.5%	10.6 +21.8%	39.8 +42.1%	422,025 +74.1%	3	20.2 +8.0%	11.4 +31.0%	45.3 +61.8%	517,500 +113.4%
17	3	21.0 +19.3%	15.7 +36.5%	36.7 +82.6%	620,400 +169.1%	3	19.5 +10.8%	19.0 +65.2%	30.0 +49.3%	557,200 +141.7%
18	4	22.1 −14.3%	28.6 +183.2%	25.3 +53.3%	845,175 +375.1%	4	20.1 −22.1%	19.6 +94.1%	20.0 +21.2%	407,350 +128.9%
19	3	27.7 +3.7%	32.5 +170.8%	22.3 +60.0%	753,800 +319.7%	3	21.0 +21.3%	24.4 +103.3%	15.7 +12.1%	442,767 +146.5%
20	3	27.7 −22.0%	31.3 +165.3%	16.3 −20.5%	513,400 −113.3%	4	22.9 −35.5%	20.3 +72.0%	14.3 −30.2%	282,150 +17.2%
21	1	29.0 0.0	46.4 +256.9%	14.0 −17.6%	649,600 +197.7%	3	24.5 −15.5%	19.5 −50.0%	16.7 −21.8%	336,500 +54.2%

* Since the sections were 5μ thick, mitoses in a 0.2 sq. mm. area also represent the number in a volume of 0.001 cu. mm. In computing the numbers of mitoses for thyroid weight in mg./100 gm. of body weight, 1 cu. mm. is considered equivalent to 1 mg.

tion of the yolk sacs in chick embryos. Methyl thiouracil is less effective in these respects than either thiourea or propyl thiouracil.

Effect of antithyroid drugs on body weights: The experiment lasted only 7 days and only one injection of an antithyroid drug was given; therefore, it was not surprising that the effects on growth as judged by body weight were relatively slight (table 1).

The average body weights of all of the treated chicks autopsied on the 15th through the 17th days of incubation were sometimes higher, sometimes lower than those of the controls. From the 18th day of incubation through the 21st those of the chicks injected with thiourea and propyl

thiouracil were consistently lower than those of the controls; those dosed with methyl thiouracil were lower on the 18th and 20th days, but higher or the same on the 19th and 21st. With each drug the greatest percentage decreases occurred on the 20th day: with thiourea, 34.1%; with methyl thiouracil, 22.0%; with propyl thiouracil, 35.5%. Since there were not enough embryos dissected each day to determine statistical significance, the body weights of each group for the entire 7-day period were pooled, giving the following figures: 18 control chicks, 22.78 ± 1.16 gm.; 23 thiourea-injected chicks, 20.64 ± 0.55 gm.; 19 methyl thiouracil injected chicks, 22.74 ± 0.79 gm.; 23 propyl thiouracil-injected chicks, 20.50 ± 0.53 gm. The difference between the weight of the control group and each of those receiving an antithyroid drug is in no case significant. However, a similar treatment of comparable data on chicks of the same breed given a single injection of 0.4% thiourea on the 14th day of incubation and killed daily thereafter showed a statistically significant difference (Adams and Bull, 1949). The figures were: 29 control chicks, 23.97 ± 1.14 gm.; 19 thiourea-injected chicks, 16.58 ± 0.69 gm. These authors also found that in series of chick embryos injected with thiourea on the 8th, or 8th and 14th, or 8th, 14th, and 18th days of incubation, the body weights at autopsy on the 21st day were consistently less than those of controls (Adams and Bull, 1949, table 1). Moreover, the tibio-tarsal lengths in their treated chicks were also less than those of their controls (Adams and Bull, table 2). Decreased body weights in New Hampshire Red chicks after doses of thiourea or thiouracil have recently been reported (McCreight, 1950). These chicks were injected on the 6th, 12th and 18th days of incubation and killed at daily intervals thereafter. Some papers, however, include no data on the body weights or growth of chicks injected with thiourea or hatched from eggs laid by hens fed thiouracil (Grossowicz, 1946; Andrews and Schnetzler, 1945; McCartney and Shaffner, 1949).

The only reports of the effects of goitrogenic agents on the body weights of chick embryos that have been found are those already mentioned. There are several on the administration of such drugs to very young chicks, a day to a week old, that have a bearing on the possible influence of such compounds on early growth. In these experiments, the antithyroid drug, thiourea or thiouracil, was included in the food or drinking water for varying periods of time and in varying quantities (Astwood, Bissell and Hughes, 1944; Mixner, Reineke and Turner, 1944; Schultze and Turner, 1945; Briggs and Lillie, 1946; D'Angelo, Gordon and Charipper, 1947). Although there is some discrepancy in the results, in general the data indicate that after long continued daily treatment animals weighed less than their controls. With 0.5% thiouracil given for several weeks beginning at hatching, cretinism resulted (Astwood, Bissell and Hughes, 1944).

One-day old chicks of several breeds usually responded to two weeks of 0.1% thiouracil in their food by slight losses in body weights, although occasionally there were gains (Mixner *et al.*, 1944, table 1). Thiouracil (0.1% to 0.8%) included in the food of White Leghorn chicks for two weeks from the day of hatching lowered the body weights approximately 10 gm. compared with the controls (Schultze and Turner, 1945, table V). Such weak solutions of thiourea as 0.05% and 0.075% caused only negligible reductions in the weight of White Rock chicks that were fed the drug for 3 weeks from hatching. However, thiourea of 0.1% strength reduced the weight of female chicks 48.8 gm. (37.2%) and of males 34.8 gm. (28.2%) below those of their controls (Schultze and Turner, 1945, table Xd). With a longer treatment of 20 weeks, 0.1% thiourea caused a 43.4% decrease in the body weights of White Leghorn male chicks, but of only 32.9% in those of females compared with their respective controls. On the other hand, after thiouracil (0.2%) given for a similar period, the percentage decreases in body weight were only 6.0% and 7.7% in males and females, respectively (Schultze and Turner, 1945, table V). There was a reduction of 27.2% in chicks of a New Hampshire-Barred Rock ×New Hampshire cross given 0.5% thiouracil in their food for 5 weeks (Briggs and Lillie, 1946). Body weights lower than those of their controls were also reported in chicks of a Barred Rock×New Hampshire Red cross fed 0.1% thiourea in the ration for 20 weeks (D'Angelo, Gordon and Charipper, 1947).

In these experiments, the general effects upon the body weight of newly hatched chicks of treatment with moderately strong solutions of antithyroid drugs were: long treatments of 3 to 20 weeks usually reduced it; short ones sometimes reduced it and sometimes did not; breeds differed in their responses; and thiourea was more effective than thiouracil. It is of interest that in this investigation as well as that of Adams and Bull (1949), a single injection of an antithyroid drug usually retarded growth within 7 days, often causing as great a percentage decrease in body weight as in hatched chicks given much larger amounts by daily administration for longer periods. Of the drugs tested propyl thiouracil and thiourea were more effective than methyl thiouracil.

Effect of antithyroid drugs on thyroid glands: Weight. The average absolute thyroid weights of chick embryos given antithyroid drugs were consistently higher than those of controls except at 24 hours after the injection and this was also true of the average thyroid weights in mg./100 gm. of body weight (table 1). In both the thiourea and propyl thiouracil treated chicks, the latter figures dropped slightly on the last two days of the experiment, but in those given methyl thiouracil it was still on the increase. In this group on the 21st day, only one chick is listed (table 1), because

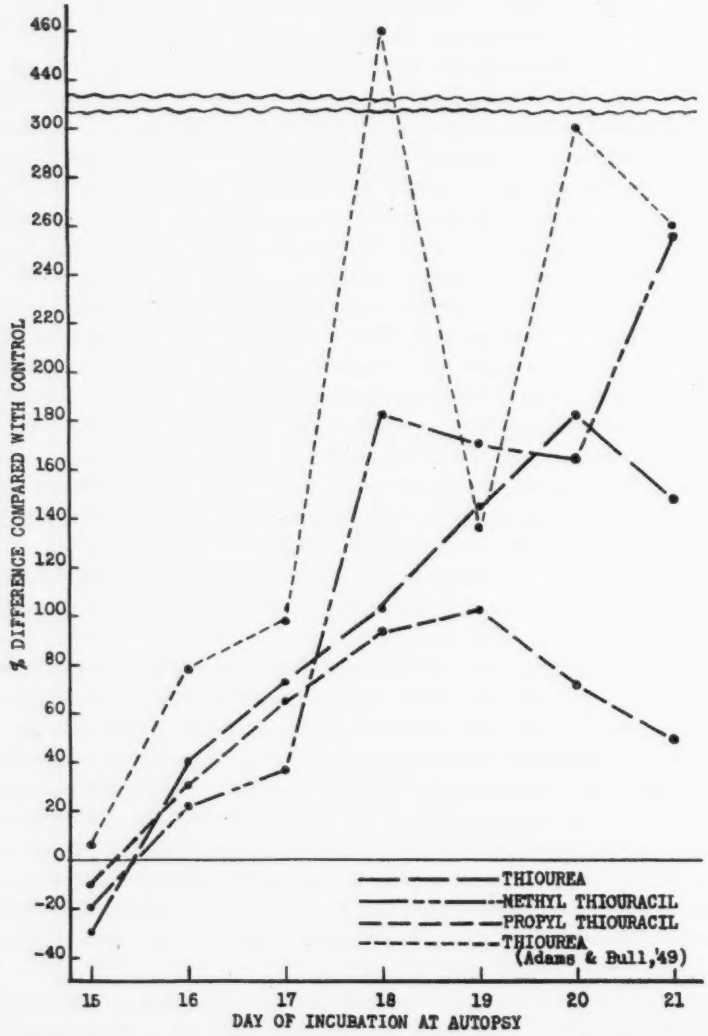

FIG. 1. Percentage differences compared with controls of thyroid weights in mg./100 gm. body weight in chick embryos injected with a single dose of an antithyroid drug on the 14th day of incubation and killed at 24 hour intervals thereafter.

the thyroids of two of the three dissected were not in good enough condition for histological study. If their data are included, the average thyroid weight for the three chicks in mg./100 gm. body weight becomes 36.4 mg. instead of 46.4 mg., which is still a higher figure than that on the 20th day (31.3 mg.). In terms of percentage increases over controls, the thyroids of the thiourea-injected embryos showed a steady rise from the 16th through the 20th day and a slight drop on the 21st; those of the methylthiouracil treated chicks, a high point on the 18th and again on the 21st; the peak for chicks that were given propyl thiouracil occurred on the 19th day and then fell (table 1, fig. 1). In nine instances, the average weights were more than doubled. It was apparent, too, that propyl thiouracil caused less increase than that produced by the other two drugs.

The foregoing data agreed fundamentally with those of a comparable series injected with thiourea on the 14th day of incubation in the experiments of Adams and Bull (1949). In fact on each day except the 19th, the percentage increases shown in their work were greater than any in the present experiments (fig. 1). In their series of chick embryos injected on the 8th, or 8th and 14th, or 8th, 14th and 18th days, the absolute thyroid weights were also increased (Adams and Bull, 1949, table 3). McCreight (1950), too, has recently obtained essentially the same results in New Hampshire Red embryos. The data also compare favorably with those of other investigators using chick embryos: the average absolute weight of the thyroids of chicks hatched from eggs laid by thiouracil-fed White Leghorn hens was approximately double that of the controls (10.48 mg. compared with 5.84 mg.; Andrews and Schnetzler, 1945); thyroids nearly three times and one and one-half times those of controls were taken from chicks hatched from eggs laid by New Hampshire Red hens fed 0.3% and 0.1% thiouracil, respectively (McCartney and Shaffner, 1949).

All the data on chick embryos subjected to antithyroid drugs have particular significance because the food supply, i.e., the yolk of the egg, does not vary. In addition in the case of embryos injected with the drugs, the dosage is known exactly. This is not quite so true of those hatched from eggs laid by hens fed thiouracil, since analysis of the eggs of such hens for thiouracil content gave a variation of 0.08–0.12 mg. thiouracil/gm. of yolk and of 0.07–0.15 mg./gm. of white (Andrews and Schnetzler, 1945). In both cases, however, the chick embryos are receiving continuous treatment since the drug is being absorbed from the yolk or/and albumen throughout the period of incubation.

In comparing the data on thyroid weight in these experiments on chick embryos with those on newly hatched chicks given antithyroid drugs, it was obvious that the increased thyroid weights in mg./100 gm. body weight in the former were not usually as high as those in day-old chicks given

antithyroid drugs continuously in the food or drinking water for 10 days (Dvoskin, 1949), or two, three, five or 20 weeks (Mixner *et al.*, 1944; Schultze and Turner, 1945; Briggs and Lillie, 1946; D'Angelo *et al.*, 1947). In such experiments, the weights ranged from three to fifteen times those of controls. One series of day-old chicks of the White Plymouth Rock breed, the same breed used in the present experiments, has particular importance. They were given 0.1% thiouracil in the diet and killed every second day through the 14th (Mixner *et al.*, 1944). The weights of their thyroids differed hardly at all from those of the controls on the second

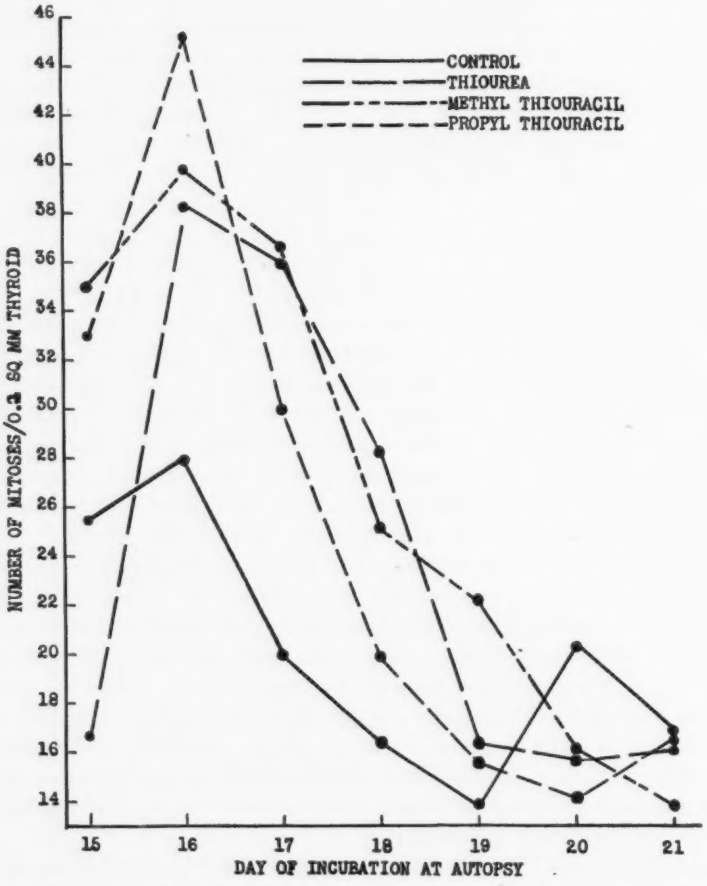

FIG. 2. Average number of mitoses in a 0.2 sq. mm. area of the thyroids of control chick embryos and of chicks injected with 0.5 cc. of an antithyroid drug on the 14th day of incubation and killed at 24 hour intervals thereafter.

day, but were definitely greater on the fourth and reached a maximum on the 12th. From their figure 2, it was estimated that on the fourth day the thyroid weight had nearly doubled and on the 12th was about seven or eight times that of the controls. The increase on the fourth day correlates well with the nearly 100% or somewhat higher increases obtained on the 18th day of incubation, i.e., on the fourth day after the injection was given, in the present experiments. However, the total amount of thiouracil received by the hatched chicks must have been considerably more than that to which the chick embryos were subjected. In still another study, chicks 48 hours old were given 0.1% thiouracil in the drinking water and killed after 3, 5, 7, 10 and 15 days (Larson, Keating, Peacock and Rawson, 1945). There was no increase in the thyroid weights until the fifth day and then only a minimal amount. This response is both less marked and slightly less rapid than those secured by Mixner *et al.* (1944), by Adams and Bull (1949), or in these experiments.

The present experiments lead to the conclusion that within a 7-day period a marked increase in the weight of the thyroid glands (mg./100 gm. body weight) of chick embryos over that in controls can be produced by a single dose of an antithyroid drug administered on the 14th day of incubation. Moreover, the increases with the three drugs tested were progressive and only began to lessen on the 6th or 7th day after the injection had been given. Both the speed and extent of the response compared favorably with those described in newly hatched chicks given continuous treatment for longer periods with antithyroid substances.

Effects of antithyroid drugs on the thyroid glands: Histology. The typical histological responses of the vertebrate thyroid to antithyroid compounds, i.e., hyperemia, colloid loss, cell hypertrophy and hyperplasia, were observed in the thyroid glands of the chick embryos given the single injection of an antithyroid drug on the 14th day of incubation (table 2). Even in embryos killed 24 hours after the injection, (i.e., on the 15th day of incubation), colloid loss was apparent, especially in the group injected with thiourea, and was probably responsible for the loss in thyroid weights on this day (table 1, fig. 1). The cells had also hypertrophied somewhat and were in general cuboidal, even high cuboidal, rather than squamous or low cuboidal as in the controls. On the 16th day of incubation, i.e., two days after the injection, these changes were even more evident and by the 18th and 19th days the cells were mainly columnar and the colloid nearly gone in all the drug-treated embryos except those given methyl thiouracil. In this group the cells were predominantly cuboidal rather than columnar and on the 19th through the 21st days of incubation, they dropped to a low cuboidal or even squamous pattern and the colloid accumulated to such an extent that it was even more abundant than in the

TABLE 2. OBSERVATIONS ON THYROID HISTOLOGY IN CONTROL CHICK EMBRYOS AND THOSE GIVEN A SINGLE INJECTION OF THIOUREA (E), METHYL THIOURACIL (M), OR PROPYL THIOURACIL (P) ON THE 14TH DAY OF INCUBATION AND AUTOPSIED AT 24 HOUR INTERVALS THEREAFTER

(For number of embryos in each group, percentage differences in thyroid weight, as mg./100 gm. body weight, between controls and drug-injected embryos, and average number of mitoses, see Table 1.)

Day killed	Treatment	Colloid*	Follicular cell pattern	Follicular size
15	C	++++	Flat to cuboidal	Small
	E	++	Cuboidal	Small
	M	+++	Cuboidal	Small
	P	+++	Cuboidal to flat	Small
16	C	++++	Cuboidal to flat	Small
	E	+	Cuboidal	Small
	M	+++	Cuboidal	Medium
	P	+	Cuboidal to columnar	Very small
17	C	++++	Cuboidal to flat	Medium
	E	++	Cuboidal to columnar	Small to medium
	M	+++	Cuboidal	Medium
	P	+	Columnar	Very small
18	C	+++++	Cuboidal to flat	Medium to large
	E	++	Columnar	Medium, small, few large
	M	++++	Cuboidal to columnar	Small, medium, large
	P	+	Columnar	Small to medium
19	C	+++++	Flat to cuboidal	Medium to large
	E	++	Columnar	Medium to large
	M	++++++	Cuboidal	Large
	P	+	Columnar	Small
20	C	+++++	Flat to cuboidal	Medium to large
	E	++	Columnar to cuboidal	Small to medium
	M	++++++	Cuboidal to flat	Large
	P	+	Columnar to cuboidal	Small
21	C	+++++	Flat to cuboidal	Medium
	E	+++	Cuboidal to columnar	Medium
	M	++++++	Cuboidal	Large
	P	+	Columnar to cuboidal	Small

* +, faint traces; ++, well depleted; +++, partially depleted; ++++, normal amount; +++++, abundant; ++++++, very abundant.

control glands. Many large follicles were also typical of these glands. On the 20th and 21st days in the group given propyl thiouracil, colloid was still scanty and the cells mainly columnar, but in the group receiving thiourea colloid was somewhat more abundant and noticeably vacuolated and many cuboidal as well as columnar cells were present. In these two groups, too, the follicular size varied, being consistently small in the thyroids of the former and mainly medium in those of the latter. In thyroids of both thiourea- and methyl thiouracil-injected chicks, parafollicular cells similar to those described by Thomas (1944) and Bull (1948) were occasionally observed.

The particular focus of the present study was the hyperplasia (increase in numbers of cells) in the thyroid glands. Counts of mitoses in comparable areas of the thyroids of drug-treated chick embryos and their controls indicated that in the glands of the methyl thiouracil and propyl thiouracil injected embryos at 24 hours after the injection the numbers of mitoses were well above those in the controls, although in chicks receiving thiourea

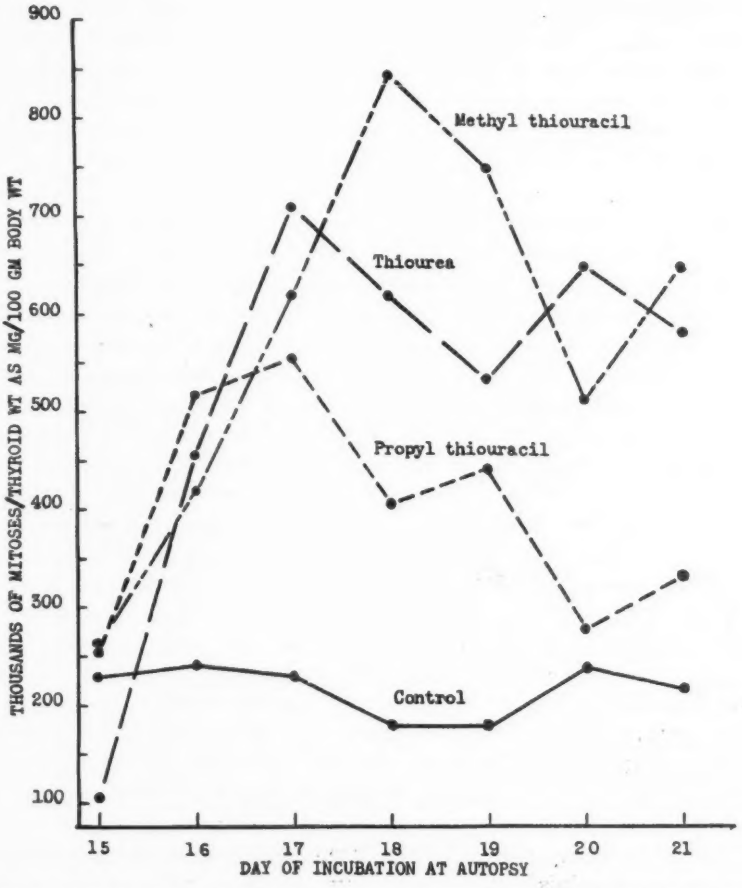

FIG. 3. Average number of mitoses in thyroids (mg./100 gm. body weight) of control chick embryos and of chicks injected with 0.5 cc. of an antithyroid drug on the 14th day of incubation and killed at 24 hour intervals thereafter. The number of mitoses in a 0.2 sq. mm. area of thyroid was assumed to represent also the number in a volume of 0.001 cu. mm. since the sections were 5μ thick, and 1 cu. mm. was considered the equivalent of 1 mg.

the count was lower (table 1, fig. 2). In all groups a peak was reached at 48 hours, i.e., on the 16th day of incubation and thereafter the numbers fell until on the 20th and 21st days, they were slightly below those in the control chicks. On the 16th day of incubation the most marked response was in the group dosed with propyl thiouracil, but by the 18th and 19th days the numbers in this group were almost at the level of the controls.

In order to take into account the comparative sizes of the thyroids, mitoses were computed for the thyroid weights in mg./100 gm. of body weight (table 1). The curves for the glands of the methyl thouracil-and thiourea-treated groups proved to be very similar and rose considerably higher than that for the embryos given propyl thiouracil (fig. 3). It was apparent that the peak in each curve was shifted to the right compared with its position when the figures on 0.2 sq. mm. areas were plotted (cf. figs. 2 and 3).

Thus, it is clear that the changes in the histological picture of the thyroids of chick embryos subjected to a single injection of an antithyroid drug on the 14th day of incubation and autopsied at 24 hour intervals thereafter were striking when compared with controls. In the glands of embryos treated with thiourea and propyl thiouracil, the stores of colloid were depleted and the follicular epithelium was heightened over the 7-day period (15th through the 21st days of incubation). The response of the epithelium was slightly weaker on the 6th and 7th days after the injection. In contrast to these, in the glands of the chicks treated with methyl thiouracil, the colloid and epithelium reacted with relative mildness on the first three days after the injection; and on the last three days, they held more colloid than the controls and the epithelium was equally low cuboidal or occasionally flat. Through the 19th day all three drugs effected a higher mitotic activity than that in controls if counts of dividing cells in comparable areas were used. But, if the mitotic activity was estimated in terms of thyroid weight in mg./100 gm. of body weight, it was higher throughout the 7 days of the experiment. On the basis of the activity in a 0.2 sq. mm. area (fig. 2), it was evident that methyl thiouracil caused the second most marked response on the 16th day; but, in terms of thyroid weight in mg./100 gm. of body weight, it was the greatest on the 18th day (fig. 3).

All of this activity resembles the intense reaction of the thyroid gland following the administration of antithyroid compounds in many animals. Reports of it have described the long-time effects of the drugs, i.e., after weeks or months of treatment. They emphasized microscopic changes in the thyroid such as hyperemia, loss of colloid, heightening of the follicular epithelium, and hyperplasia, though usually in its very general sense rather than its precise meaning of cellular proliferation. Mitoses

have rarely been mentioned although the marked increase in the weight of the thyroid as well as the increase in the number of follicles sometimes cited both imply cell division. There are some reports of the histological picture of the thyroid after short periods of treatment, a few hours or days, and in these loss of colloid and increased follicular cell height have been fully described and the latter often measured but there is no mention of cell numbers. In fact only one paper on mitotic activity after treatment with antithyroid drugs has been discovered in the literature. In this paper no mitotic counts are given (Paschkis, Cantarow, Rakoff and Rothenberg, 1945). It is only stated that the number of dividing cells in the thyroid glands of rats increased as early as 24 hours after the thiouracil had been added to the drinking water and, as administration of the drug continued, the number of mitoses became markedly high at 5 days, rose to a maximum at 10 days and diminished strikingly after 15 days. The authors did not observe any constant relation between the thinning of the colloid and increased cell height *and* the stimulation of mitoses, but in some cases the former preceded the mitotic activity. This study is particularly relevant to the present experiments in that it interprets increased cell division as an early response to an antithyroid drug. However, the animals used were mammals. Moreover, since the treatment was continuous, they must have been subjected to relatively much larger amounts of the antithyroid drug than the chick embryos. These factors may account for the peaks in number of mitoses occurring after many days of treatment.

One other paper refers to mitotic activity in the thyroid following a relatively short treatment with an antithyroid drug (Lever, Miltenburg and van Oordt, 1949). The work was done on fishes, the freshwater *Lebistes reticulatus* and the marine *Callionymus lyra*. The authors utilized the method devised by Lever (1948) for determining the activity of thyroid glands. They reported that the thyroids of the fishes kept in solutions of 0.01% and 0.1% thiourea showed within 7 days a marked heightening of the follicular epithelium, but that "cell divisions have not taken place." This conclusion does not tally with that of Paschkis *et al.* (1945) or with the results here reported. It may be that stimulation of mitoses by an antithyroid drug occurs later in a cold-blooded animal such as the fish, than in warm-blooded ones. The lizard, *Anolis carolinensis*, injected daily with thiourea for 21 days and killed after one, three, seven, 14 and 21 doses, first showed marked cellular hypertrophy in its thyroid after seven doses (Adams and Craig, 1951). According to Gasche (1946), however, the thyroid epithelium of *Xenopus* tadpoles changed characteristically only 24 hours after administrating an antithyroid drug. Other studies on cold-blooded animals given antithyroid drugs give no pertinent information on

early thyroid response (Charipper and Gordon, 1947; Goldsmith, 1949; Joel, Gordon and Charipper, 1949; Lynn and Wachowski, 1951; Ratzersdorfer *et al.*, 1949). To be sure, if counts in the present investigation had been made only on the 7th day after the injection and if the data for comparable areas, e.g., 0.2 sq. mm., in the glands of drug-treated and control embryos had been compared, a somewhat similar conclusion might have been reached. However, it would have been corrected when the mitoses were estimated for the thyroid weight in relation to body weight.

In view of the results of these experiments, the possibility that cell division may be stimulated in an already enlarged gland in the hyperthyroid human being given antithyroid drugs should be examined. In reports of the effects of such drugs in cases of thyrotoxicosis, none was found that included data on early biopsy examinations. In several hyperplasia was mentioned but only after treatment had been continued for considerable time and the term appeared to be used in its general rather than specific sense (Astwood, 1943; Williams and Bissell, 1943; Halpert, Cavanaugh and Keltz, 1945).

According to the interpretation of the action of antithyroid drugs, an inhibition of the synthesis of the thyroid hormone causes its reduction in the blood and this stimulates the production of more thyrotrophic hormone by the anterior pituitary. Both the hypertrophy and the hyperplasia in the thyroids of the chick embryos studied may be attributed to the effect of this hormone. It is pertinent, therefore, to inquire whether these responses are similar to and as prompt as those stimulated by the administration of thyrotrophic hormone. In the majority of such studies, treatments have been long continued and the emphasis has been on the colloid loss, the striking increase in cell height and on thyroid weight rather than on the mitotic activity (Adams, 1946). For example, after direct injection of chick embryos at 12 to 20 days of incubation with two doses of 0.2 ml. of thyrotrophic hormone, Woodside (1937) observed completely activated thyroids containing high epithelium and no follicular colloid 24 hours after the injection, but he made no reference to numbers of dividing cells. In others that he injected on the 10th or 11th day of incubation "complete activation was evident up to the 18th day" and on the 19th day "the number of mitoses, although rather large was still within the number frequently seen in untreated controls" (p. 426). Schockaert (1932) mentioned enlargement and hyperplasia of the cells in the thyroids of young ducks seven days after the administration of bovine anterior pituitary suspension and stated that the number of mitoses in the experimental glands was higher than that in controls.

In some of the earliest of such experiments on mammals, Loeb and Bassett (1930) injected guinea pigs with 1.0 cc. of acid or alkaline extract of

cattle anterior pituitary substance daily for one to 7 days killing animals at 24 hour intervals. They found that the hypertrophy of the thyroid epithelium was apparent even after one injection and that it was increased to a maximum by the 5th and 6th days. The peak of mitotic activity, however, occurred at the end of the 2nd or 3rd day; after that the numbers of dividing cells dropped. They emphasized that "the maximum increase in mitotic activity is reached before the stage of maximum hypertrophy and absorption of colloid" (p. 491). Likewise Aron (1930a, b) discovered that an extract containing 0.33 gm. of fresh beef pituitary induced a 100% thyroid weight increase, many mitoses and colloid depletion and that the cells multiplied rapidly during the first days of the treatment.

Cogent and more recent reports are those of Keating, Rawson, Peacock and Evans (1945), of Larson, Keating, Peacock and Rawson (1945), and of Rawson (1949). Keating *et al.*, found a steady increase in acinar cell height as well as in thyroid weight for 5 days in Red Rock cockerels given Antuitrin T daily for 4 days and killed at 24 hour intervals. They attributed the "disproportionate increase in thyroid weights" to "a true cellular hyperplasia." With a single dose of thyrotrophic hormone and autopsy every 24 hours, both acinar cell height and thyroid weight rose at 24 and 48 hours after the injection; at 72 and 96 hours, both had decreased markedly and the weight was almost that of the controls. Considering that the early increase in cell height is accompanied by a loss of colloid the added thyroid weight must be in part due to more cells which also hypertrophy.

The part of a report by Larson *et al.* (1945) relevant to this discussion compares the effects of thiouracil and of thyrotrophic hormone on the anatomic changes in the chick's thyroid. Chicks 48 hours old given 0.1% thiouracil in the drinking water were killed at 3, 5, 7, 10 and 15 days; others of the same age were given either $\frac{1}{4}$ U. or 1 U. of thyrotrophic hormone in distilled water and killed at 1, 3, 7 and 10 days. Larson and his coworkers found that with thyrotrophic hormone the mean acinar cell height was much increased over that in controls by 24 hours (with $\frac{1}{4}$ U. to 4.7 μ and with 1 U. to 4.2 μ compared with 1.5 μ in controls). It leveled off at 7.9 μ and 10.1 μ, respectively, by the 10th day. With thiouracil, after 72 hours the height was only 1.9 μ compared with 1.5 μ in controls. On the 5th day, it was 3.1 μ; on the 7th, 6.5 μ; on the 10th, 8.9 μ; and on the 15th, 10.4 μ. According to these figures, the cellular hypertrophy in the thyroid was less prompt after thiouracil than after the thyrotrophic hormone. The increase in the weight of the thyroid following thiouracil administration was also slower. With thyrotrophic hormone, while little or no change occurred during the first 24 hours, there was a marked increase by the end of the third day. With thouracil, there was no increase

in thyroid weight until the 5th day and then only a minimal one. In quality, the response of the chick thyroid to thyrotrophic hormone and to thiouracil was strikingly similar. However, it began later after thiouracil and only became as strong as that to thyrotrophic hormone after some days of treatment. Larson *et al.*, therefore, suggest that the lag in the response with thiouracil was due to the necessity for colloid discharge before the thyroid hormone insufficiency was great enough to stimulate increased production of thyrotrophic hormone and hence thyroid cell hypertrophy and hyperplasia.

Rawson (1949) reported a response in cockerels 5 days of age, injected with 1 U. of thyrotrophic hormone, some of which were killed every 6 hours for a 48-hour period. It was found that loss of thyroid iodine occurred 6 hours after the injection of pituitary hormone with a maximum loss at 24 hours. The mean cell height did not change until 18 hours after the injection and reached its peak at 30 hours. No mention was made of mitoses. Rawson pictures the events in the thyroids of animals treated with thyrotrophic hormone as occurring in the following sequence: loss of thyroid iodine or thyroid hormone; an increase in acinar cell height; an increase in thyroid weight; and an increased capacity to concentrate iodine.

These studies of Rawson and his colleagues suggest that the increased mitotic activity, i.e., true hyperplasia, that will contribute largely to the increased thyroid weight, occurs subsequently to the loss of colloid and the heightening of the follicular epithelium in the thyroid either when stimulated directly by thyrotrophic hormone or indirectly by it after thiouracil. They emphasize that the response to the latter is slower though eventually just as pronounced. The present study and also some unpublished data on the thyroids of chick embryos, show that colloid loss, cell hypertrophy and increased cell division all may occur within 24 hours after an injection of an antithyroid drug. They further indicate that the increase in thyroid weight is due not only to the hypertrophy of the cells present at the beginning of the experiment but also to that of the multitude of daughter cells.

Varying activity of the antithyroid compounds used: In the present experiments on chick embryos, it was evident that the three antithyroid compounds acted somewhat differently. Propyl thiouracil induced the most intense response in the thyroid in terms of evacuation of colloid, of maintenance of hypertrophy of the follicular epithelium and of increase in numbers of mitoses in a given area (0.2 sq. mm.) on the 16th day (tables 1, 2; fig. 2). However, it was less effective than methyl thiouracil or thiourea in the 7-day period of the experiment when the number of mitoses was calculated on the basis of the thyroid weight in mg./100 gm. of body weight or when the percentage increase of the thyroid weight over that of controls

was considered (figs. 1, 3). Thiourea ranked second in causing loss of colloid and increase in cell height, but it and methyl thiouracil were much alike in the number of mitoses found in comparable areas on consecutive days through the 21st day of incubation. If mitoses were figured on the thyroid weight basis, methyl thiouracil seemed a little more effective than thiourea (fig. 3), but in percentage increase of thyroid weight over controls, they were nearly the same (fig. 1). In the thyroids of the methyl thiouracil injected group, the striking difference really concerned the histological picture. The colloid release and increase in cell height were much less than with the other two drugs. The increase in thyroid weight appeared to be due largely to the secretion and storage of colloid and to the large size of the follicles (table 2). Hatching, yolk sac retraction and body weight were not affected by this drug. In this connection Kopf, Loeser and Meyer (1948) found that methyl thiouracil caused a loss in weight of the pituitary and a decrease in the production of thyrotrophic hormone in rats which could be interpreted as indicating that it was less effective than some others of the antithyroid drugs.

Several other reports reveal differences in the action of antithyroid drugs. In man, 6-methyl thiouracil was twice as active and 6-n propyl thiouracil 0.75 times as active as thiouracil (Stanley and Astwood, 1947). In the Rhesus monkey, propyl thiouracil was one half as effective as thiouracil and methyl thiouracil which were equal in their capacity to modify the degree and rate of the accumulation of radioactive iodine (McGinty and Wilson, 1949). In rats n-propyl thiouracil was shown to be more potent than methyl thiouracil (Astwood, 1945; Bavin and Goodchild, 1946). Thiouracil was more effective as an antithyroid compound than thiourea in rats and man, whereas in chicks thiourea was equal to (Mixner *et al.*, 1944) or more potent than thiouracil (Astwood, Bissell and Hughes, 1945; Schultze and Turner, 1945; VanderLaan and Bissell, 1946; Adams and Bull, 1949; Lever, 1949; McCreight, 1950) or methyl thiouracil (Lever, 1949). Thiouracil was also nearly twice as effective as 6-propyl thiouracil in chicks (Gassner *et al.*, 1950). Thus it seems likely that the goitrogenic potency of different antithyroid substances depends on many variables such as species and individual differences, differences in chemical structure which may affect their rate of absorption and even the details of their action on the synthesis of the hormone by the thyroid cells (Pitt-Rivers, 1950).

SUMMARY

By counting mitoses, an attempt was made to discover how early and to what extent hyperplasia, i.e., cell proliferation, occurred in the thyroids of animals treated with antithyroid drugs. White Plymouth Rock chick

embryos were given a single injection of thiourea or methyl thiouracil or propyl thiouracil on the 14th day of incubation. These and their controls were killed at intervals of 24 hours through the 21st day of incubation.

Counts of mitoses in comparable areas showed that cell proliferation began as early as the cellular hypertrophy and colloid loss in the thyroids of the drug-treated embryos. At 24 hours after the injection of two of the drugs, methyl thiouracil and propyl thiouracil, the average numbers of mitoses exceeded that in the controls. With all three of the drugs, a peak in mitotic activity was reached at 48 hours. By the 21st day of incubation, the average numbers of mitoses in the thyroids of drug-treated and control embryos was approximately the same. However, if mitoses were calculated for thyroid weights in mg./100 gm. of body weight, in order to take into account the differences in thyroid weights between drug-injected embryos and their controls, it was evident that mitotic activity remained higher than that in the controls throughout the seven-day period of the experiment.

The increase in the weight of the thyroids of the chick embryos treated with the antithyroid drugs was due not only to the hypertrophy of the follicular cells present in the glands when treatment began but also to the marked increase in the numbers of cells by mitosis and to their subsequent hypertrophy.

Judged by the degree of cellular hypertrophy, hyperplasia and colloid release in the chick thyroids, the three antithyroid compounds, thiourea, methyl thiouracil and propyl thiouracil, have somewhat different goitrogenic properties.

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ON EXTRA-ADRENAL ACTIONS OF ADRENOCORTICOTROPHIC PREPARATIONS

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IT HAS been shown recently that ACTH (Connaught) increases the weight of the preputial glands of the rat, by virtue of a non-adrenal-mediated action (Jacot and Selye, in press). This same ACTH preparation also proved to exert another extra-adrenal effect, in that it induced thymolysis, though only in the presence of threshold doses of cortisone (Selye, 1951). Both these actions are quite independent of the steroid hormone producing glands themselves, since they were demonstrable even in animals whose adrenals and gonads had been removed.

In order to explore the possibility that the above-mentioned actions were not due to the ACTH itself, but to some contamination present in our extract, we have now repeated these experiments with a different ACTH preparation. The latter (kindly supplied by Professor E. B. Astwood) proved to be much more active than the commercially available ACTH (Astwood, Raben, Payne and Grady, 1951), both as regards its clinical efficacy and as judged by its ability to deplete the ascorbic acid content of the rat adrenal after hypophysectomy.

We naturally also had to consider the possibility that contamination with gonadotrophins may be responsible for the preputial gland stimulating effect. However, as we shall see, the ACTH preparations were capable of stimulating the preputial glands even after adrenalectomy and gonadectomy, while under these conditions purified gonadotrophins no longer exhibited this effect.

EXPERIMENTAL PROCEDURE

Three experiments were performed on a total of 150 female piebald rats.

First experiment.—80 rats were bilaterally adrenalectomized and ovariectomized, 48 hours before the initiation of the injections. Immediately after this, they were given 1% NaCl instead of tap water as a drinking fluid. The removal of the ovaries and adrenals was necessary in order to eliminate both of the known endogenous sources of steroid hormones.

These rats were divided into 8 groups as indicated in Table I.

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All the controls for ACTH-treated animals were injected with *hypertensinogen* in order to determine the possible effect of a non-hormonal foreign protein. It was given at a daily dose level of 0.6 mg. in the form of four subcutaneous injections of 0.15 ml. of a suspension containing 1 mg. per ml. in olive oil.

ACTH (Astwood) was given at two different daily dose levels: 0.15 mg. and 0.6 mg. in the form of four subcutaneous injections of 0.15 ml. of suspensions containing 0.25 mg. and 1 mg. per ml. of oil.

Cortisone was administered in a single subcutaneous injection of 0.5 mg. per day, in the form of microcrystalline suspension (as distributed by Merck and Co. Limited, Montreal, Canada) containing 5 mg. of cortisone acetate per ml.

Chorionic gonadotrophins (A.P.L. distributed by Ayerst, McKenna and Harrison Ltd., Montreal, Canada) were given at a daily dose level of 50 I.U. in a single injection of 0.1 ml.

Prolactin (International Standard) was given at a daily dose level of 0.17 I.U. (0.017 mg.) in a single injection of 0.1 ml.

Second experiment.—40 intact rats were divided into four groups as indicated in Table II.

These treatments were applied in the same manner and at the same dose level as in the above experiment.

Third experiment.—30 intact rats were divided into five groups, treated during six days as indicated in Table III.

Hypertensinogen was administered for the same reason as in the previous experiments. It was given at a daily dose level of 1.2 mg. in the form of six subcutaneous injections of 0.2 mg. in 0.1 ml. saline solution, every four hours.

ACTH (Connaught Laboratories, Toronto, Lots 4-2) was given in the same manner as hypertensinogen, at four different daily dose levels. 0.1 ml. of the different concentrations of ACTH in saline (Group II: 10 mg. per ml., Group III: 5 mg. per ml., Group IV: 2 mg. per ml., and Group V: 1 mg. per ml.) was injected subcutaneously six times a day at four hour intervals, to ensure a constant hormone supply. It will be noted that in the first two experiments ACTH was given only at six hour intervals, since, being suspended in oil, its absorption rate is delayed.

At the end of the experiments, all animals were killed by exsanguination, after which their preputial glands and thymuses were removed for weighing.

EXPERIMENTAL RESULTS

To facilitate the perusal of our results, we summarized the principal data in three tables, each corresponding to one of the experiments above.

1.—As seen in Table I, 0.6 mg. per day of the purified ACTH (Astwood) increases the weight of the preputial glands in adrenalectomized and ovariectomized rats as much as 6 mg. per day of ACTH (Connaught) (Astwood *et al.*, 1951); this increase is highly significant since "P" between groups I and IV or V is <0.01. However, ACTH (Astwood) has no thymolytic effect in the presence of threshold doses of cortisone as evidenced by comparing Groups III and V with Group VI.

2.—The administration of graded doses of ACTH (Connaught) to intact rats (Table III) revealed that 0.6 mg. per day suffices to cause an unquestionable enlargement of the preputial glands ("P" between Groups

TABLE I. EFFECT OF A SIX DAY TREATMENT WITH DIFFERENT PITUITARY HORMONES IN ADRENALECTOMIZED AND OVARIECTOMIZED RATS

Groups	Treatment	Body weight in gm.		Weight of the preputial glands		Weight of the thymus	
		Initial	Final	mg.	mg./100 gm. B.W.	mg.	mg./100 gm. B.W.
I	Hypertensinogen	96	98	27.1	27.6 ± 2.16	303	307 ± 18
II	ACTH (low dose)*	95	96	32.7	33.8 ± 1.75	332	343 ± 11.7
III	ACTH (low dose) + Cortisone	93	102	37.6	36.5 ± 2.08	246	236 ± 13.3
IV	ACTH (high dose)†	95	97	41.8	42.2 ± 3.38	288	299 ± 22.3
V	ACTH (high dose) + Cortisone	96	104	40.5	39.3 ± 2.64	211	203 ± 7.7
VI	Hypertensinogen + Cortisone	95	105	31.3	29.9 ± 0.89	219	207 ± 12.9
VII	Chorionic Gonadotrophins	97	101	31.7	30.6 ± 4.53	289	283 ± 7.4
VIII	Prolactin	96	98	25.5	26.0 ± 2.02	287	295 ± 15.7

* 0.15 mg. per day.
† 0.6 mg. per day.

TABLE II. EFFECT OF A SIX DAY TREATMENT WITH DIFFERENT PITUITARY HORMONES IN INTACT RATS

Groups	Treatment	Body weight in gm.		Weight of the preputial glands		Weight of the thymus	
		Initial	Final	mg.	mg./100 gm. B.W.	mg.	mg./100 gm. B.W.
I	Hypertensinogen	88	104	36.4	34.8 − 2.2	243	233 − 20.0
II	ACTH (0.15 mg. per day)	88	106	57.4	54.3 ± 3.3	113	104 ± 15.3
III	Chorionic Gonadotrophins	87	106	65.6	61.7 ± 2.68	212	199 ± 12.6
IV	Prolactin	87	106	39.4	36.9 ± 3.5	245	231 ± 12.1

TABLE III. EFFECT OF A SIX DAY TREATMENT WITH DIFFERENT DOSES OF ACTH IN INTACT RATS

Groups	Treatment	Body weight in gm.		Weight of the preputial glands		Weight of the thymus	
		Initial	Final	mg.	mg./100 gm. B.W.	mg.	mg./100 gm. B.W.
I	Hypertensinogen	76	86	35.7	41.9 ± 3.76	199	227 ± 23.2
II	ACTH (6 mg. per day)	77	77	74.1	95.4 ± 14.0	49	64 ± 6.9
III	ACTH (3 mg. per day)	77	81	58.2	71.1 ± 5.34	74	92 ± 8.8
IV	ACTH (1.2 mg. per day)	76	88	60.6	70.1 ± 11.0	145	164 ± 22.3
V	ACTH (0.6 mg. per day)	76	89	53.0	58.7 ± 3.83	177	196 ± 17.1

I and V <0.01) but it induces no thymolysis. 3 mg. are required to obtain a significant action on the thymus ("P" between Groups I and IV >0.05, is not significant).

3.—Chorionic gonadotrophins are most potent in stimulating the preputial glands of normal rats (Table II). Yet, they have no effect on these glands in adrenalectomized and ovariectomized rats (Table I).

4.—Prolactin failed to stimulate the preputial glands either in normal or in adrenalectomized and spayed rats.

DISCUSSION

Even a purified ACTH preparation induced an enlargement of the preputial glands in the absence of adrenals. However, it failed to exhibit the thymolytic action which we had previously obtained with a less pure ACTH extract in adrenalectomized rats maintained on small doses of cortisone. It is possible that the purified extract is devoid of the thymolytic principle. It is perhaps more probable, however, that, at low dose levels, the hormone sensitivity of the preputial glands is greater than that of the thymus. If this interpretation were correct, it would be unnecessary to introduce the hypothesis of a separate "thymolytic factor." Perhaps we used the purified ACTH at a dose level just adequate to cause preputial-gland stimulation, but insufficient to induce any detectable thymolysis.

Such a difference in target organ receptivity is not without precedent in endocrinology. It will be recalled that testosterone, which possesses both folliculoid and testoid actions, exhibits one or the other effect predominantly, at different dose levels (Selye, 1949).

The third experiment appears to support the view that such a difference in target organ receptivity also exists as regards ACTH. In fact a very small dose of ordinary ACTH is sufficient to cause definite preputial gland stimulation, in intact rats, without inducing any thymolysis. As the dose level is raised, thymus involution becomes increasingly more evident.

It is also evident that the preputial gland stimulation obtained by ACTH (Connaught) in both adrenalectomized and ovariectomized rats is not due to contaminating gonadotrophins. The latter are notoriously effective in stimulating the preputial glands of intact animals, but, as the present experimental series indicates, they exhibit no such effect in rats deprived of their adrenals and gonads.

The possibility that the preputial gland stimulating effect of our ACTH preparation might have been due to contamination with prolactin has also been outruled, since this hormone is entirely devoid of this property under any circumstances.

SUMMARY

The previously observed extra-adrenal preputial gland stimulating action of commercial ACTH preparations have been reproduced by a purified ACTH extract in female, adrenalectomized and ovariectomized rats. At the dose level used in the present experimental series, this ACTH preparation failed, however, to cause thymolysis in adrenalectomized rats maintained on small doses of cortisone.

Furthermore, as judged by our data, the preputial gland stimulating effect exhibited by ACTH preparations after adrenalectomy and gonadectomy should not be ascribed to gonadotrophins.

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SOME EFFECTS OF CORTISONE ON VITAMIN C-DEFICIENT GUINEA PIGS

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INTRODUCTION

A RELATIONSHIP between vitamin C and functioning of the adrenal cortex has been recognized but some phases of the nature of the interaction are still in question. Reports of this relationship have been given by Lockwood and Hartman (1933), Lockwood *et al.* (1936), Kuchel and Mitchell (1936), Lauber and Rosenfeld (1938), Broch (1939), Giroud and Martinet (1940), Hartman (1941), Prina (1946), Tuba, Hunter and Osborne (1946), Dean and Morse (1948), Vogt (1948), and Stepto, Pirani, and Consolazio (1950). To study a phase of this relationship, guinea pigs were fed a diet deficient in vitamin C, some of which received cortisone and the others did not.

MATERIALS AND METHODS

In this study, 20 male guinea pigs, each weighing approximately 280 grams, were used. All animals were given the same basal diet: hulled oats, 75 lbs.; soy bean meal, 25 lbs.; Lederle fortified supplement, ½ lb.; animal protein factor which contained 2.5 grams of vitamin B_{12} per lb., ½ lb.; vitamin A, 56,000 I U; vitamin D, at least 1½ times the amount for minimum maintenance. In addition alfalfa pellets, in which the vitamin C had been destroyed by heat and drying, were given. The animals were kept in a room maintained at a temperature ranging from 68° to 73° Fahrenheit.

RESULTS

Eight of the guinea pigs (Group I) were given the vitamin C-free diet only. All animals exhibited pain about the joints after 11 days on the diet and all were dead after 19 days. Survival time on the C-free diet ranged from 12 to 19 days. Before death typical conditions of scurvy developed as presented by Meyer and McCormick (1928), and Dann and Cowgill (1935). As part of the scorbutic changes these guinea pigs developed bone deficiencies similar to those described by Israel and Frankel (1926), Hanke (1936), Harman, Kramer and Kirgis (1938), Lexer (1939),

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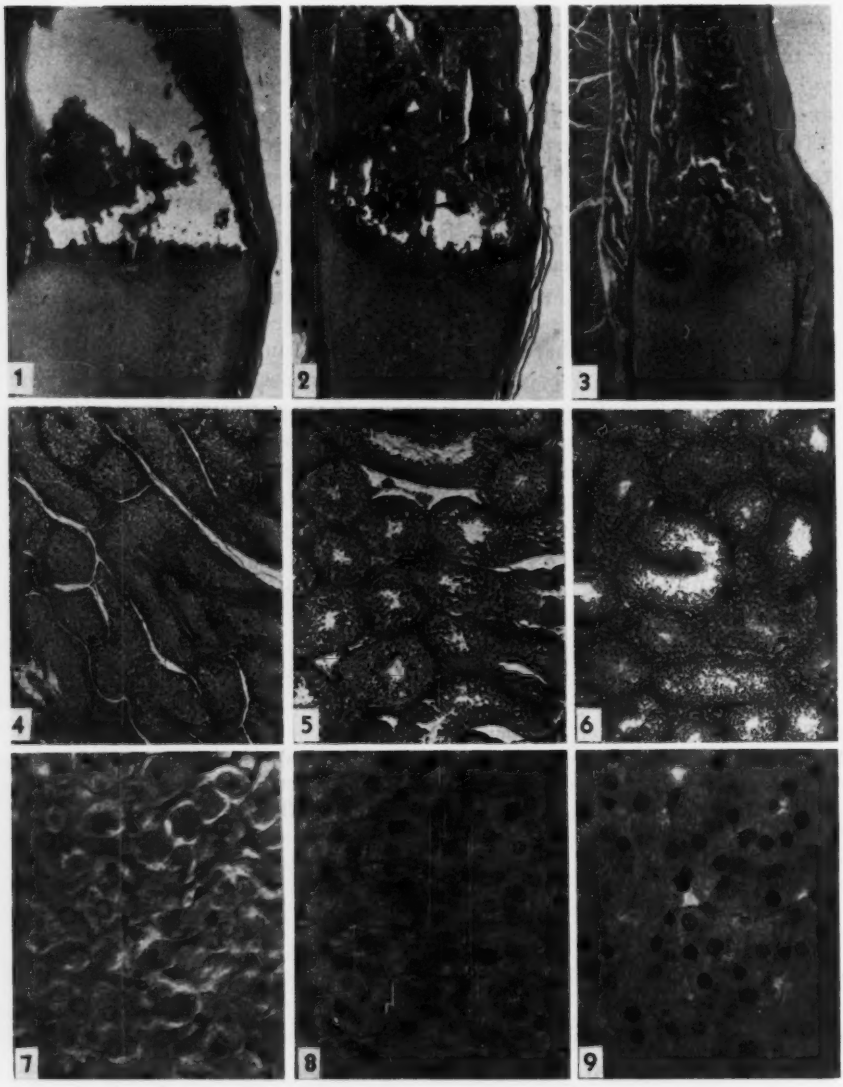

FIG. 1. Cartilage—bone junction of rib from guinea pig on scorbutic diet (Group I) showing much degeneration of bone.

FIG. 2. Cartilage—bone junction of rib from guinea pig on scorbutic diet but treated with cortisone (Group II) showing only slight bone degeneration.

FIG. 3. Bone—cartilage junction of rib from guinea pig on an adequate diet (Group III).

FIG. 4. Testis from scorbutic guinea pig (Group I) showing degeneration of the semi-

Bourne (1942), and Hyman, Ragen and Turner (1950). Histological preparations of ribs and heel joints of all animals were made and studied. Little bone tissue other than some of the perichondrial type remained (Fig. 1). Further scorbutic changes included degeneration of the testes involving a breakdown in structure of the seminiferous tubules. These changes were similar to those described by Lindsay and Medes (1926), Harman and Gillum (1937), and Harman (1950). The seminiferous tubules in this group averaged 100 μ in diameter and the walls of the tubules (if lumina were present) averaged 24 μ in thickness. The average weight of the testes was .315 gm. In many tubules a lumen was not distinguishable and there was no evidence of spermatogenesis. Figure 4 is a photomicrograph of a testis from a C-free animal and is typical of all animals in the group.

Adrenal glands from animals deprived of vitamin C were similar to those in scorbutic guinea pigs described by Hoerr (1931), Meyer and McCormick (1928) and Morikawa (1920) (Fig. 7). The cortical cells become enlarged but there were degenerative changes with vacuolation and disintegration of cells. There was an increase in the lipoid content in the zona fasciculata. The presence of many mitotic figures at the junction of the zona glomerulosa and zona fasciculata confirms the work of Iwabuchi in 1922 as reported by Hoerr (1931).

Eight other guinea pigs (Group II) were given the same diet as Group I, but 5 days after they were first placed on the diet they were given 5 mg. of cortisone[2] daily by subcutaneous injection. Three of these guinea pigs were killed at times corresponding to survival times of the animals in Group I. The other five were kept for survival time. They lived from 37 to 50 days, an average of 46 days, on the C-free diet plus cortisone. They continued to gain weight for 23 days then lost until death. Weight loss following their maximum weight averaged 58 gm. each.

Animals from this group that were killed, to compare with those from Group I that were dying, appeared normal in every respect. Those taken after the end of the survival time of Group I exhibited only slight altera-

[2] The cortisone acetate used in this study was supplied by Merck and Company, Rahway, N. J.

niferous tubules.

FIG. 5. Testis from guinea pig on scorbutic diet but treated with cortisone (Group II) showing nearly normal condition of the seminiferous tubules, 10 days after all of Group I had died.

FIG. 6. Testis from guinea pig on an adequate diet (Group III).

FIG. 7. Adrenal cortex from scorbutic guinea pig (Group I).

FIG. 8. Adrenal cortex from guinea pig on scorbutic diet but treated with cortisone (Group II), showing cells that are not actively secreting.

FIG. 9. Adrenal gland from guinea pig on an adequate diet (Group III).

tion of bone as indicated in Figure 2. Testes from Group II guinea pigs averaged .662 gram in weight. Seminiferous tubules measured 120 micra in total diameter and the walls were 31.1 micra in thickness. Analysis of the measurements of the tubules indicated there was a difference of high statistical significance ($P = <.0001$) between Groups I and II. Spermatogenesis was in progress and mature sperm were present (Fig. 5). All animals in Group II were similar regarding bone and testis maintenance.

In Group II the adrenal cortical tissue showed alteration from the normal but typical of conditions found in animals treated with cortisone as reported by Hyman, Ragan and Turner (1950) and Winter, Silber, and Stoerk (1950). The cells of the zona glomerulosa were small and their nuclei were small. Few cytoplasmic granules were observed (Fig. 8).

In Group III, 6 guinea pigs were given the same diet as Groups I and II except that ascorbic acid in adequate supply was provided. These animals made usual gains in weight and appeared normal in every way. Photomicrographs of bone (Fig. 3), testis (Fig. 6), and adrenal tissues (Fig. 9) from these normal control animals are presented.

SUMMARY

1. Guinea pigs from which vitamin C was witheld developed the usual conditions of scurvy with painful joints and degenerating bones and testes. Death occurred from 12 to 19 days after the pigs were placed on the scorbutic diet.

2. Other guinea pigs that received no vitamin C but were given 5 mg. of cortisone daily lived from 17 to 45 days on the scorbutic diet and developed no painful joints. Bone structure remained nearly normal and the testes remained almost indistinguishable from those of normal animals.

3. Guinea pigs that received cortisone developed adrenal glands with atrophied cells, shrunken nuclei, few cytoplasmic granules, and decreased lipoidal vacuolation.

4. Guinea pigs fed the same diet as the others, but with ascorbic acid added, were normal in all respects.

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CYTOCHEMICAL STUDY OF THE ADRENAL CORTEX OF THE RAT WITH EXPERIMENTAL CARDIAC HYPERTROPHY

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CHARACTERISTIC responses of the adrenal cortex to variations in the sodium: potassium relations of the blood were observed by several workers. It has been demonstrated that low Na/K ratios in the blood induce a characteristic hypertrophy of the zona glomerulosa of the adrenal cortex (Bacchus, 1950a, 1950b; Deane, Shaw, and Greep, 1948; Nichols, 1948). Conversely, influences tending to augment the Na/K ratio lead to signs of atrophy and hypofunction of this zone of the adrenal. The selective zona glomerulosa hypertrophy following KCl flooding (Bacchus, 1951), and sodium deficiency (Deane, Shaw, and Greep, 1948), is prevented by simultaneous DCA treatment. These findings support the hypothesis that the glomerular zone is the site of production of mineralocorticoid hormones which serve to maintain a suitable Na/K ratio in the body.

Na/K balance and the adrenal cortex have been implicated in the etiology of cardiovascular and renal lesions. It seemed desirable to investigate the action of the hypertension-producing Grollman operation on the structure of the adrenal, and on the response of the gland to potassium chloride flooding.

MATERIALS AND METHODS

Five groups of 10 female Wistar rats each were used in this study. Group I consisted of normal intact rats which were kept on an ad libitum fare of tap water and Purina Laboratory Chow. Groups II and III consisted of rats which were submitted to one-stage Grollman operations (1944). The operation involved placing a figure-of-eight-ligature around the left kidney and extirpation of the right kidney. One week after the operation, Group II rats were placed on a fare of tap water and Chow, Group III rats being exposed to potassium chloride flooding. Potassium chloride flooding involved an ad libitum fare of Chow and a 2% aqueous solution of potassium chloride as the sole source of fluid. Groups IV and V were unilaterally nephrectomized, and one week post-operatively were placed on the dietary fares as Groups II and III respectively. Forty to forty-four days after submission to the dietary fares all animals were killed. The adrenals, hearts, and kidneys were extirpated and weighed. The adrenals were fixed in 10%

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neutralized formalin. Frozen sections (15μ) of one adrenal of each animal were made and stained with Sudan IV and for Schultz-positive material as described in a previous work. Some sections were immersed in acetone for 15 minutes and then stained as above. The remaining adrenal of each animal was embeddded in Fisher's Tissuemat and sectioned 6μ thick. These paraffin sections were stained with Delafield's alum-hematoxylin and eosin. (Bacchus, 1950a).

In order to study changes in the widths of the zones of the adrenal cortex the following procedure was applied. An ocular micrometer disc was placed in the 5×ocular lens of the microscope and the slides examined under 10×objective lens; the ocular micrometer disc was previously calibrated against a standard. These measurements were made at four cardinal points of three Sudan-stained sections of one adrenal of each animal. The sections were taken from the middle portions of the adrenals.

RESULTS

Observations made at weekly intervals indicated that the body weights of the animals were not appreciably affected by the treatments. The organ weight data are presented in Table 1. It is observed that the heart weights of the animals with Grollman-operated kidneys were significantly higher than those of normal intact, and unilaterally nephrectomized, control rats. Potassium chloride flooding to these animals failed to depress the cardiac

TABLE 1. EFFECT OF GROLLMAN OPERATION AND UNILATERAL NEPHRECTOMY ON ORGAN WEIGHTS OF RATS ON TAP WATER, AND ON POTASSIUM CHLORIDE FLOODING

Group & No. Rats	I (10)	II (10)	III (10)	IV (10)	V (10)
Surgery	None	Grollman*	Grollman*	Unil. nphx.	Unil. nphx.
Dietary fare	Chow & H_2O	Chow & H_2O	Chow & KCl	Chow & H_2O	Chow & KCl
Body Weight. g ±S.D.**					
Initial	169 ± 3	172 ±23	163 ±17	163 ±19	173 ±13
Final	212 ±24	220 ±21	205 ±21	214 ±12	218 ±19
Kidney Wt. g/100g ±S.D	0.79 ±0.09 (pair)	0.65 ±0.11	0.79 ±0.10	0.58 ±0.05	0.68 ±0.07
Heart Wt. mg./100g ±S.D.	297 ±30	358 ±39	364 ±36	295 ±19	296 ±24

* Grollman operation = figure-of-eight-ligature around left kidney and extirpation of right kidney.

** S.D. = $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$

hypertrophy due to the Grollman operation. Previous work indicated that KCl flooding opposes the cardiac hypertrophic action of DCA overdosage, and depresses the heart weights of normal intact rats (Bacchus, 1951a, 1951b). As in previous studies, the kidneys of all animals under potassium chloride flooding showed greater weights than those on tap water. Potassium chloride flooding had this action even in rats whose kidneys were ligatured. The adrenals of all animals with Grollman kidneys were signifi-

cantly higher than those of the unilaterally nephrectomized, and of the normal intact, rats.

Histological data: Data on zone widths are presented in Table 2. Also presented in the Table are data on the width of the zona glomerulosa as per cent of the width of the cortex as a whole. The increased adrenal weight in the rats of Group II was due to a widening of the entire cortex. The per cent increase of the zona glomerulosa however, is in proportion to the in-

TABLE 2. ADRENAL WEIGHTS AND WIDTHS OF ADRENAL CORTEX ZONES IN GROLLMAN-OPERATED, AND UNILATERALLY NEPHRECTOMIZED RATS, WITH AND WITHOUT KCl FLOODING

Group	Treatment	Adrenal Wt. mg./100 g ±S.D.	Widths of zones (μ)			z. glom.**
			"Inner zones"*	sudanophobic z.	zona glomerulosa	Cortex %
I	None (normal)	25.3±3.2	765	17	60	6.7
II	Grollman+H_2O	33.1±7.0	1020	0	73	6.7
III	Grollman+KCl	32.2±6.3	1173	0	133	10.2
IV	Unil. Nephrex+H_2O	26.9±5.5	1071	0	56	5.0
V	Unil. Nephrex+KCl	27.2±4.8	1139	0	117	9.3

* "Inner zones" = zona fasciculata plus zona reticularis.
** Width of the zona glomerulosa as percent of width of the entire cortex.

crease in the width of the gland. The lipid content of the zona glomerulosa and of the inner zones (zona fasciculata and zona reticularis) was increased moderately. Hematoxylin and eosin sections showed no signs of mitoses in these glands. Glands of Group III rats showed signs of hypertrophy of the inner zones and of the zona glomerulosa. The glomerulosal hypertrophy was relatively greater than the increase in the inner zones, and its acetone-soluble Schultz-positive lipid complement was considerably increased. The sudanophobic zone disappeared from these glands. The histological and histochemical appearance of the glands of Group IV rats was similar to that of Group II, that is, there was cortical hypertrophy associated with a moderate increase of the content of Schultz-positive lipids. The sudanophobic zone was also absent from the adrenals of Groups IV and V rats. It becomes apparent that the increase noted in the glomerulosal width of the Group II (Grollman plus H_2O) animals is only in strict proportion to the increase in the entire cortex. Potassium chloride flooding in such animals induces a selective enlargement of the glomerular zone, this zone becoming 10% of the width of the entire cortex.

DISCUSSION

The acetone-soluble, Schultz-positive, lipids observed in the adrenals are presumed to represent the sites of steroid hormone production in the adrenal cortex (Deane, Shaw, and Greep, 1948; Bacchus, 1950a). The

data suggest that the adrenals of all Grollman operated rats were producing hormones at an increased rate. The marked increase in the Schultz positive lipid content of the zona glomerulosa cells of the potassium chloride flooded rats also suggests an increased production of steroid hormone. Since this zone is presumed to be the site of production of mineralocorticoids, it seems that this response was of a compensatory nature intended to maintain the Na/K ratio. Although the entire adrenal cortex width of the Grollman operated rats fed with tap water increased markedly, the glomerulosal width was not selectively increased. As the percentage width data indicate, the increase in glomerulosal size was in strict proportion to the general increase in cortex width. Deane and Masson (1951) reported a similar increase in the width of the glomerulosa, but failed to measure the width of the rest of the cortex.

In view of the marked increase of heart weight in the Grollman operated rats and of cardiac hypertrophy ascertained by histological study, it is probable that the operation was effective in producing some of the changes it is intended to produce (Grollman, 1944). Since there is a marked correlation between the level of blood pressure and cardiac mass, the latter measurement has been suggested as a reliable index of blood pressure (Rytand, 1938; Schroeder, 1942). Whereas it has been observed that KCl flooding prevents the cardiac damage due to mineralocorticoid excess (Bacchus, 1951a; Peschel, Black-Schaffer and Schlayer, 1951), similar treatment is ineffective in Grollman operated rats.

Whether the glomerulosal changes noted in this study indicate a marked alteration in sodium:potassium metabolism is not definite. It is possible that the Grollman operation induced these changes either directly through the secretion of renin (Deane and Masson, 1951), or indirectly through the sodium loss which renin is supposed to induce. The observation that the glomerulosal changes in the Grollman-operated water-fed rats were similar to those in the unilaterally nephrectomized controls suggests that the changes cannot be ascribed solely to renin. Certain studies have indicated that the hypertension following such kidney operations may not be associated with marked alterations in the Na/K relations (Friedman, 1948). It is not unexpected therefore, to find that rigid sodium restriction failed to alleviate the hypertension in such animals (Selye and Stone, 1949).

It may be suggested that possible variations in the Na/K ratio induced by the operation failed to be clearly and selectively reflected on the adrenal glomerular zone because of some inertia in the zone due to the kidney damage. This is improbable in view of the observation that KCl flooding into such animals induced a selective enlargement of the zona glomerulosa in excess of the general enlargement of the cortex due to the stress of the operation.

SUMMARY

The action on the cytochemical appearance of the adrenal cortex of the Grollman operation for producing hypertension has been studied. The data indicate that the operation produces cardiac hypertrophy as well as a general enlargement of the zones of the adrenal cortex. Potassium chloride flooding to such animals fails to alter the cardiac hypertrophy but produces a selective enlargement of the zona glomerulosa of the adrenal. The increased glomerulosal width observed in Grollman operated rats was not selective.

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NOTES AND COMMENTS

THE *IN VIVO* INACTIVATION OF ESTRADIOL BY THE HUMAN LIVER

INTRODUCTION

Van Wagenen and Gardner (1950) and Hooker, Drill and Pfeiffer (1947) have shown that monkey liver does not inactivate estrogens in vivo. In the human subject, it has been reported that increased levels of estrogens in both urine and blood are associated with liver disease (Glass, Edmondson and Soll, 1940, Gilder and Hoagland, 1947; Rakoff and Gross, 1951) while on the other hand, human liver slices *in vitro* inactivate estrogens to a much smaller extent than does rat liver (Twombly and Taylor, 1942).

We have studied estrogen inactivation by human liver in a way similar to that used by Golden and Severinghaus (1938) in the rat. Our results, which parallel theirs, are described below.

EXPERIMENTAL PROCEDURE AND RESULTS

Sixteen women, after complete ovariectomy, were divided as follows. Five were given no hormonal replacement therapy. Four were implanted with a 25.0 mg. estradiol[1] pellet each in the rectus muscle incision. Seven had either one (5 women) or two (2 women) 25.0 mg. estradiol pellets each implanted between the leaves of the mesentery where they separate to surround the ileum.

Vaginal cornification in the patients with either rectus muscle or mesenteric implants is compared in Figure 1. The differences indicate a marked lack of activity of the mesenteric implants in all but one patient. In this case the pellet may have slipped out and become attached to the body wall, or a vascularized adhesion between the gut and body wall may have formed.

Between 5% and 20% cornification up to the fifth postoperative month was seen in one control patient, while another showed 5% cornification until the eighth postoperative week when the cornification percentage rose to and stayed at 40%–50% until the fifteenth week. This is especially unusual since this patient complained of moderate menopausal symptoms throughout the period of study. One smear each from a third control patient in her third and from a fourth in her fifteenth postoperative week showed 0% cornification. No smears could be obtained from the fifth untreated patient.

Menopausal symptoms were absent in all the patients with rectus muscle implants. Moderate to severe symptoms[2] began in the control group between the third and seventh postoperative week and continued until the third to fifth months, when observations were either discontinued, or the patients were put on oral estrogen therapy. Five of the seven patients with mesenteric implants had moderate to severe symptoms, which began between the first and fourth postoperative week and continued for at least five weeks in one, and for between ten weeks and four months in the others. Of the two who showed no symptoms, one exhibited a high level of vaginal cornification (see above), and the other had had a natural menopause eight years prior to surgery, and was free of symptoms when this study was begun.

DISCUSSION

These findings would indicate that estradiol absorped into the hepatic portal system in women exercises little estrogenic effect. The absorption of estradiol directly into the

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[1] We are indebted to Dr. Edward Henderson of the Schering Corporation for the generous supplies of estradiol used in this study.

[2] If hot flashes occurred at the rate of about once per day, but did not arouse the patient from sleep, symptoms were designated as moderate. If they occurred more often than this, and aroused the patient from sleep, they were designated as severe.

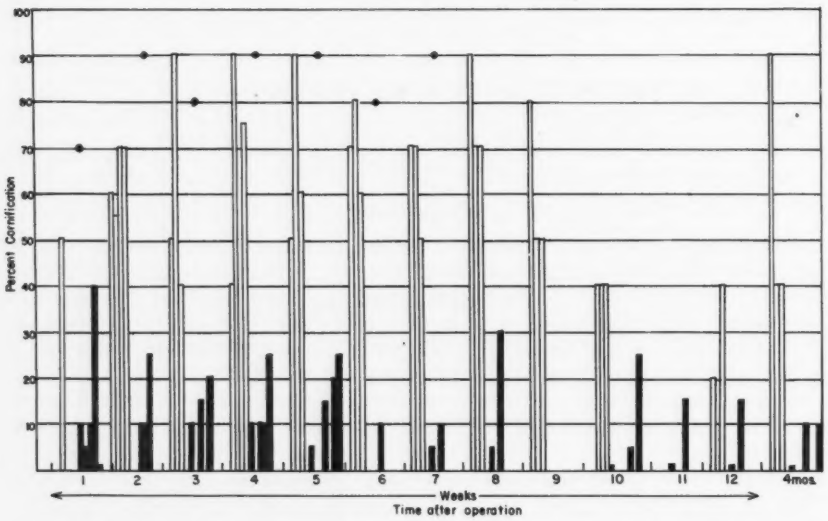

FIG. I. *The degree of vaginal cornification in patients ovariectomized and bearing pellets of estradiol in either the rectus muscle incision or the mesentery.* Each open column represents a patient with a rectus muscle implant, each black column a patient with a mesenteric implant. Degree of cornification is indicated by the height of the column in each case. The order of placement of the columns at each postoperative interval is always the same. The circles represent one patient in the mesenteric implant group who showed a high level of vaginal cornification and no menopausal symptoms throughout the period of study.

systemic circulation, however, is associated with definite evidences of estrogenic activity. In this respect, the human liver is essentially similar to the rat's, rabbit's, guinea pig's, and dog's, but is entirely different from the monkey's. The latter thus stands relatively alone in the inability of its liver to inactivate estrogens, although the mechanisms whereby systemically injected estrogens are inactivated are as effective as they are in other species studied (Westerfeld and Doisy, 1946).

SUMMARY

Seven ovariectomized women with mesenteric estradiol implants showed little vaginal cornification, and a high incidence of menopausal symptom up to four months postoperatively, while four similar women, with rectus muscle estradiol implants, showed a high level of vaginal cornification and no menopausal symptoms up to seven months postoperatively.

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EXCRETION OF ESTROGENS AND A METABOLITE IN THE URINE OF FEMALE PSEUDOHERMAPHRODITES

In the course of a study of a fluorogenic substance occurring in the neutral fraction of the urine of two female pseudohermaphrodite patients (Zondek and Finkelstein, 1951) the estrone and estriol content of the phenolic fraction was investigated.

The estrogen determinations were done fluorometrically (Finkelstein, 1947, 1948, 1951) and were checked by bioassay (Allen-Doisy Test).

Table 1 summarizes the results obtained by both methods.

TABLE 1. URINARY EXCRETION OF ESTRONE AND ESTRIOL IN FEMALE PSEUDOHERMAPHRODITES AND IN NORMAL SUBJECTS DETERMINED FLUOROMETRICALLY AND BY BIOASSAY

Subject	Diagnosis	Estrone fluor. γ/liter	Estrone bioassay* γ/liter	Estriol fluor. γ/liter	Estriol bioassay** γ/liter
N.K. f.	pseudohermaphrodite	120	20	150	100
M.H. f.	pseudohermaphrodite	100	20	180	60
female	normal	mean 10	mean 10		
male	normal	mean 17	mean 3		

* 1 MU = 0.2γ estrone.
** 1 MU = 1γ estriol (in oil).

It is evident from the experimental data obtained by bioassay and by fluorometry, that both estrone and estriol were excreted by the patients at much higher levels than were found in normal subjects.

The absolute values obtained for estriol fluorometrically were slightly higher than those obtained by bioassay. On the other hand, the values for estrone differed greatly; the comparison of data obtained fluorometrically and by bioassay was striking.

Thus, the fluorometric estimation of estrone indicated about 100γ per liter of urine, whereas bioassay registered only about 100 MU which corresponds to about 29γ estrone. (1 MU in our mice is equivalent to about 0.2γ estrone).

The ratio of estrone measured fluorometrically F (100γ) to estrone determined by bioassay B (20γ) was F:B = 5:1. The ratio obtained by the comparison of fluorometric and bioassay data on the urine of normal men (Table 1) was also about 5:1. On the other hand, in the urine of normal women no difference was found in the values for estrone obtained by both methods and the F:B ration was 1:1. (Table 1).

It was concluded from these observations that the urine of normal males and also that of female pseudohermaphrodites contains a fluorogenic substance in the estrone fraction differing from estrone. This substance may be a metabolite of estrogen as was suggested in the case of male urine by Engle (1950).

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THE EFFECT OF IODOTHIOURACIL ON THE BIOLOGIC DECAY OF THYROIDAL RADIOIODINE

Gassner and associates (1950) have presented evidence that 5-iodo-2-thiouracil has an action on the thyroid which is different both quantitatively and qualitatively from that of thiouracil or from that of thiouracil to which an equivalent amount of inorganic iodide has been added. The goitrogenic effect of iodothiouracil as measured by thyroid weight, hyperplasia, vascularization, cell height, and colloid droplets was less than that of an equimolar quantity of thiouracil. In rats, the iodine content of the thyroid was not as depleted as after administration of thiouracil (Barrett, *et al.*, 1951). Another difference was that iodothiouracil did not potentiate the effect of thyrotropin, an action readily brought about by thiouracil (Albert, *et al.*, 1947). However, the antithyroid activity of both iodothiouracil and thiouracil, as measured by depression of the basal metabolic rate, or by blocking thyroidal uptake of I^{131}, appeared equal. These results indicate that some degree of separation between goitrogenicity and antithyroid activity has been induced by iodothiouracil. In view of this, it seemed desirable to investigate the antithyroid activity of iodothiouracil by further procedure. Accordingly, we have examined its effect on the biologic decay of thyroidal I^{131}.

The biologic decay of thyroidal radioiodine reflects the secretory rate of the thyroid (Albert, 1951; Wolff, 1951; Perry, 1951). Rats (males of the Sprague-Dawley strain) maintained on an iodine-deficient diet are injected with 1 to 2 microcuries of radioiodide. Beginning on the second day after injection, *in vivo* counts over the thyroidal area are made daily for a period of about two weeks. The daily counts corrected for physical decay are then plotted on semilogarithmic paper, and the observations so obtained for each animal are visually fitted with a straight line. The proportional rate of disappearance of thyroidal I^{131} is then determined from this curve in terms of per cent loss per day. Since virtually all of the thyroidal I^{131} is protein-bound, the rate of loss of I^{131} from the thyroid presumably reflects the rate of discharge of labeled organic iodine, or hormone. It has been shown previously that the secretion rate as determined in this manner is markedly inhibited by hypophysectomy (Randall, *et al.*, 1951) and very greatly increased by administration of a goitrogenic drug such as thiouracil (Albert and Tenney, 1951).

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In the first experiment the effect of administering iodothiouracil orally was determined. Twelve rats maintained on an iodine-deficient diet were injected with 1 microcurie of radioiodide. Six of the animals were maintained on an iodine-deficient diet as controls; the other 6 were maintained on the same diet to which 1 per cent iodothiouracil had been added. The secretory rate (mean $\pm$ standard error) of the control animals was $7.76 \pm 0.75\%$ per day, whereas that for iodothiouracil was $44.64 \pm 4.86\%$ ("t" = 7.5). The increase in the secretory rate was of the same order of magnitude as that induced by thiouracil (Albert and Tenney, 1951).

A second experiment was carried out to compare more precisely the antithyroid activity of iodothiouracil and thiouracil. Thirty rats prepared with radioiodine as described were separated into five groups. They were maintained on the iodine-deficient diet and injected subcutaneously with the following daily doses: group A, controls; group B, 10 mg. thiouracil; group C, 20 mg. iodothiouracil; group D, 10 mg. iodide as potassium iodide; group E, 10 mg. thiouracil $\pm$ 10 mg. iodide as potassium iodide. The secretory rates were: group A, controls $8.01 \pm 0.77\%$; group B, thiouracil $38.56 \pm 1.19\%$; group C, iodothiouracil $59.16 \pm 4.68\%$; group D, potassium iodide $12.64 \pm 1.19\%$; group E, thiouracil+iodide $33.22 \pm 2.52\%$. The "t" values of the various groups receiving goitrogens as compared to the controls indicated a high level of significance; namely, 22, 11 and 10 for thiouracil, iodothiouracil and thiouracil plus iodide, respectively.

Thus, the antithyroid activity of iodothiouracil as measured by the loss of thyroidal I^{131} is as great as that of thiouracil on an equimolar basis. If this is the case, the relative inability of iodothiouracil to deplete thyroidal I^{127}, reported by Barrett, *et al.* (1951), probably cannot be ascribed to a weak antithyroid action. Instead, it may perhaps represent accumulation of iodothiouracil itself or of some iodine-containing metabolite of iodothiouracil within the thyroid while the gland is losing its own stored hormone.

SUMMARY

The antithyroid activity of iodothiouracil as measured by its effect on the biologic decay of thyroidal I^{131} in the rat is as great as that of thiouracil.

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DETECTABLE AMOUNTS OF ADRENOCORTICOTROPHIC HORMONE IN BLOOD FOLLOWING EPINEPHRINE

Epinephrine depletes adrenal ascorbic acid and cholesterol (Long and Fry, 1945), induces an eosinopenia (Recant *et al.*, 1948) and increases the concentration of cortical hormone in adrenal vein blood (Vogt, 1944). These effects of epinephrine are mediated by adrenocorticotrophic hormone (ACTH) (Sayers, 1950), which is presumably discharged at an accelerated rate in response to the injection of the drug. In the present study, the blood of epinephrine-injected animals has been assayed for ACTH in recipient hypophysectomized rats; the results indicate that epinephrine increases the titre of ACTH in the blood.

METHODS

In the first experiment, a technique of blood transfusion was used. Male rats were anesthetized with ether and given an intravenous injection of synthetic l-epinephrine bitartrate in 0.5 cc. of a 0.9 per cent solution of NaCl made to pH 2 with HCl. The injection of the solution occupied 30 seconds.

After a fixed interval, 3 cc. of blood were quickly removed from the external jugular vein and immediately transfused into the corresponding vein of a recipient hypophysectomized rat for the assay of ACTH by the method of Sayers *et al.* (1948). The difference in ascorbic acid concentration between the left adrenal of the assay rat, removed just before the transfusion, and the right adrenal removed one hour later was taken as an index of the ACTH in the blood of the donor animal.

The rats were males of the Sprague-Dawley strain, weighing 120–160 gm. The recipient rats were hypophysectomized 22–26 hours before the assay and were given 5 per cent glucose to drink following the operation. Completeness of hypophysectomy was determined by examining the sella under 9 times magnification. Ascorbic acid was analyzed by the method of Mindlin and Butler (1937).

Fifteen groups of 8 rats each were divided among all possible combinations of 5 doses of epinephrine (0 (control), 1.25, 2.5, 5.0 and 10 μg.) and 3 bleeding times (1, 4 and 8 minutes). Five experiments were conducted in which 5 μg. of epinephrine were added in vitro to 3 cc. of normal rat blood and assayed for ACTH activity as described above.

The second part of the experiment was designed to determine if the ACTH activity present in blood after epinephrine administration could be concentrated by the method of Gemzell *et al.* (1951). The method is a modification of that used by Lyons (1937) to obtain hypophyseal prolactin. Pooled lots of blood were obtained as follows: one minute after the intravenous injection of 5 μg. of epinephrine in acid saline, 4 or 5 cc. of blood were quickly withdrawn from the external jugular vein and immediately introduced into a flask immersed in ice. One hundred cc. of blood were collected from 20 rats. Heparin (0.025 mg. per cc. blood) was used as the anticoagulant. An equal quantity of blood was obtained in the same way from controls injected with acid saline. The blood was centrifuged, the plasma removed and acid acetone (25 cc. of concentrated HCl per liter C. P. acetone) was added slowly to the plasma with stirring until the acetone concentration was 80 per cent. After an hour, the precipitate was separated by centrifugation and redissolved in a volume of distilled water equal to the original plasma volume. The solution was brought to 80 per cent acetone concentration as before and immediately centrifuged. The two supernatants were combined and poured into a volume of absolute acetone sufficient to bring the final concentration of acetone to 96 per cent. A fine white precipitate formed overnight; the supernatant was discarded and the precipitate dried under vacuum. The entire procedure was carried out at 3° C. The next day the dried precipitates were redissolved in a few cc. of 0.01 N NaOH and the volume adjusted so that 1 cc. of extract was equivalent to 15 cc. of blood. The solutions were kept at 0° C. during the assay for ACTH. Two dose levels (equivalent to 5 and 15 cc. of blood) were assayed against two dose levels (0.3 and 0.9 milliunit[1]) of ACTH [Armour's lot 60–61, equivalent in potency to International Standard ACTH (La-1-A)]. Two such experiments were carried out separately.

RESULTS AND DISCUSSION

Blood from control animals given saline had no effect on the adrenal ascorbic acid of the hypophysectomized recipient rats. Blood from untreated rats to which epi-

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[1] One International Unit of ACTH is the activity of 1.0 mg. of the International Standard La-1-A. One milliunit is equal to 0.001 Unit.

nephrine had been added in vitro also had no effect. Three cc. of blood taken from epinephrine-injected rats induced a significant depletion of adrenal ascorbic acid when injected intravenously into hypophysectomized assay rats (Table 1). This may be interpreted as indicating the presence of ACTH in the blood. The effect is demonstrable

TABLE 1. DEPLETION OF ADRENAL ASCORBIC ACID FOLLOWING THE TRANSFUSION OF 3 CC. OF BLOOD FROM EPINEPHRINE-INJECTED RATS INTO HYPOPHYSECTOMIZED ASSAY RATS

Time between epi. inj. and blood withdrawal in minutes	Ascorbic acid depletion, mg./100 gm. adrenal tissue				
	0	1.25	Dose of epinephrine, μg. 2.5	5.0	10.0
1	6 ± 5	50 ± 9*	37 ± 8*	45 ± 7*	56 ± 10*
4	13 ± 6	24 ± 9	28 ± 2*	70 ± 11*	60 ± 9*
8	9 ± 7	33 ± 8*	18 ± 6	35 ± 4*	40 ± 7*

Each value represents the mean and standard error of 8 assay animals. Values indicated by (*) are significantly different from the appropriate time controls. (Student's t test, $p < .05$).

TABLE 2. ASSAY OF ACETONE-PRECIPITATED FRACTION OF RAT BLOOD FOR ACTH ACTIVITY

	Blood source				ACTH Standard*	
	Epinephrine-treated rats		Control rats			
Volume injected	0.33 cc.	1.0 cc.	0.33 cc.	1.0 cc.	0.3 milliunit	0.9 milliunit
Equivalent volume of blood	5 cc.	15 cc.	5 cc.	15 cc.		
Response (depletion of adrenal ascorbic acid, mg./100 gm. adrenal)	51 44 45 45 28 23 44	114 71 93 147 94 94 110 69	17 0 5 7 12 1 2	51 67 72 41 53	81 36 42 29 49 34	84 95 108 81 85 76
Average	40.0	99.0	2.8	56.8	45.1	88.1
s	19.7		10.4		15.7	
b	123.6		113.1		90.2	
λ	0.159		0.092		0.174	
M $\frac{\text{treated blood}}{\text{control blood}}$	0.3633					
$\pm s_M$	0.0761					
Potency $\frac{\text{treated blood}}{\text{control blood}}$	231 ± 40%					
M $\frac{\text{blood}}{\text{ACTH}}$	$\bar{2}.8057$		$\bar{2}.4425$			
$\pm s_M$	0.0632		0.0624			
Potency blood in milliunit per cc.	0.064 ± .009		0.026 ± .0004			

s combined = 16.14 b combined = 110.0

b = slope of the $\log_{10}$ dose response curve; s = standard deviation of all the individual responses about this curve; λ = s/b and is a measure of the accuracy of the assay method; M = logarithm of the ratio of potencies; s_M = standard error of M.

* Armour's ACTH lot 60–61, equivalent in potency to International Standard ACTH (La-1-A), *i.e.*, 1.0 mg. of 60–61 equals 1.0 International Unit of ACTH activity.

within one minute after epinephrine injection. The level of ACTH activity appeared to decline by 4 minutes after 1.25 and 2.5 μg. doses had been given and by 8 minutes after 5.0 and 10.0 μg. doses.

ACTH activity was demonstrated in acid-acetone precipitates of plasma from both control and epinephrine-injected animals (Table 2). The acid-acetone precipitates equivalent to 5 and 15 cc. of blood were assayed against 0.3 and 0.9 milliunit ACTH standard. Since the slopes of all three dose response curves did not differ significantly it was possible to express the potency of blood in terms of standard. It was estimated that the control blood contained 0.026 milliunit ACTH per cc. and blood from the epinephrine-treated rats contained 0.064 milliunit per cc.

SUMMARY

Blood from control and epinephrine-injected rats was assayed for ACTH in recipient hypophysectomized rats. The ACTH titre was demonstrated to be increased following epinephrine injection. The ACTH in blood from control rats was estimated to be 0.026 milliunit per cc. One minute after epinephrine administration, the concentration of ACTH in blood was estimated to be 0.064 milliunit per cc. It is concluded that the mechanism which controls the blood level of ACTH responds with extreme rapidity to the administration of epinephrine.

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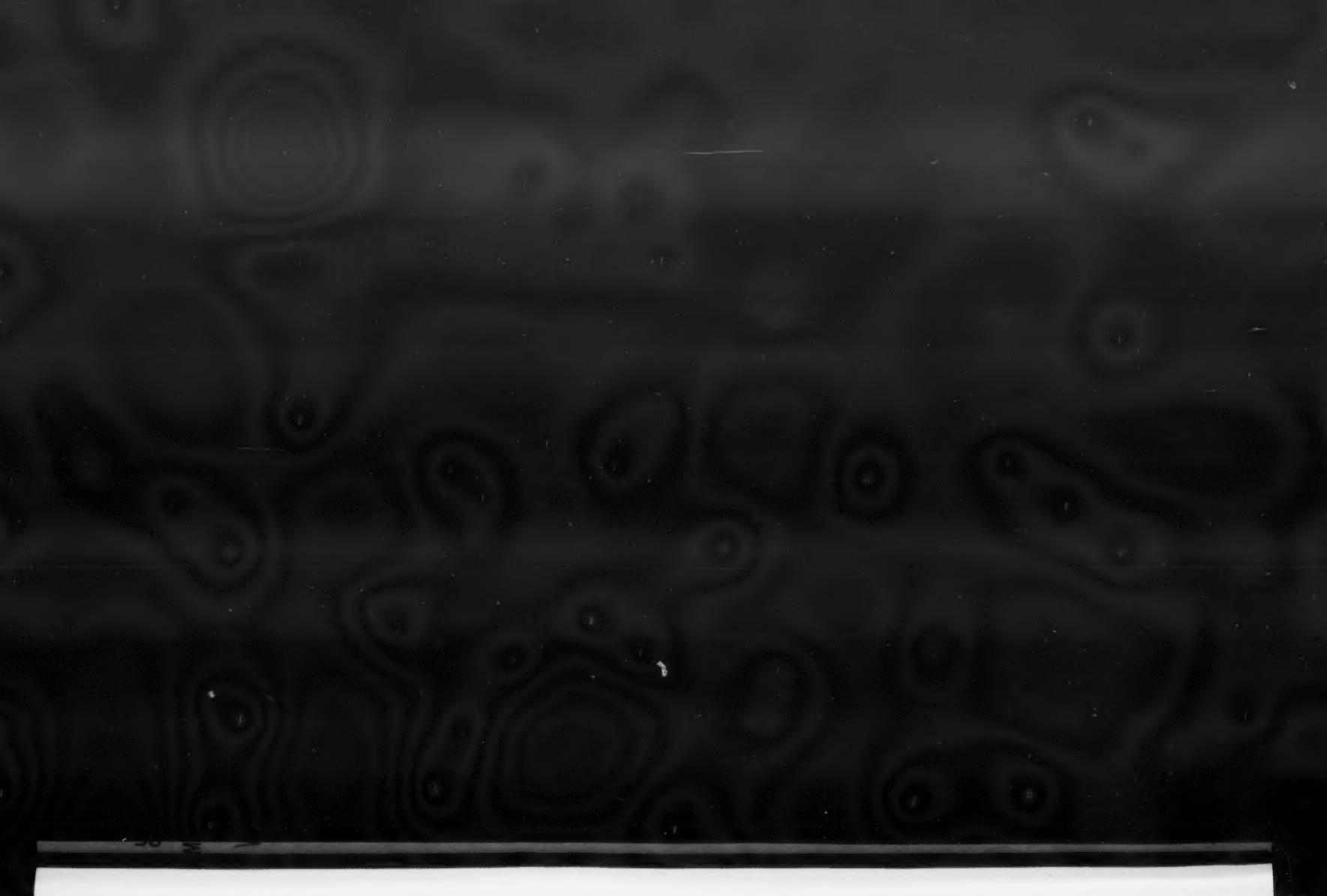
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